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The role of C5a in the development of thrombotic glomerulonephritis in rats

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SUMMARY

Thrombus formation is the important pathologic finding observed in glomerulonephritis induced by antiglomerular basement membrane (GBM) antibodies. Although strong deposition of C3 and membrane attack complex (MAC) is observed in this disease, the role of complement has not been fully elucidated. The aim of this work was to investigate the role of complement, especially an anaphylatoxin C5a, in a rat model of thrombotic glomerulonephritis. Rats were first pretreated with subclinical dose of lipopolysaccharide (LPS). Thrombotic glomerulonephritis was then induced by intravenous injection with rabbit antirat GBM (RbAGBM) (Group I). For the evaluation of the role of complement, the soluble complement receptor type 1 (sCR1) (Group II) or the C5a receptor antagonist peptide (C5aR-AP) (Group III) was intravenously administered 30 min before RbAGBM injection. For exploring the role of neutrophils, rats were pretreated with cyclophosphamide before induction of disease (Group IV). All rats were sacrificed at 6 h, and histological examination was performed.

Rats in Group I developed severe glomerular thrombosis. Leucocyte accumulation and strong binding of C3 and MAC were observed in the glomeruli. In rats treated with sCR1 (Group II) and C5aR-AP (Group III), both leucocyte accumulation and thrombus formation in the glomeruli were significantly inhibited. C3 and MAC were negative in the glomeruli in Group II rats, while they were strongly observed in Group III. In neutrophil depleted rats (Group IV), there was also deposition of C3 and MAC in the glomeruli but thrombus formation was not observed. These findings indicated that glomerular thrombosis is dependent on the leucocytes, and mediated in part by the anaphylatoxin C5a but not MAC in the present model.

Keywords anti-GBM antibody lipopolysaccharide complement anaphylatoxin leucocyte

INTRODUCTION

Thrombus formation in the glomeruli is often observed in the various types of glomerular diseases in human, and it is thought to be the important pathological process of glomerular injury [1]. Although glomerular thrombus formation is characteristically observed in the immune mediated glomerular diseases such as Goodpasture syndrome [2] and lupus nephritis [3], little is known about its pathogenesis. Since there is strong binding of C3 and membrane attack complex (MAC) in the glomerular capillaries of these patients, complement is supposed to play some roles in the development of glomerular injury. During the activation process of complement, potent anaphylatoxins such as C3a and C5a are generated. C5a has potent effects to recruit neutrophils, and to induce respiratory burst of neutrophils [4]. It also affects on the endothelial cells to upregulate procoagulant activities [5], and promote aggregation of pletelets [6]. Thus, complement activation products, especially anaphylatoxin C5a, is supposed to contribute to the thrombus formation.

To elucidate the role of complement and anaphylatoxins, we have now several tools including soluble complement receptor type 1 (sCR1) [7] and C5a receptor antagonist peptide (C5aR-AP), a synthetic hexapeptide (NMePhe-Lys-Pro-dCha-Trp-dArg) [8]. sCR1 efficiently inhibits complement at the level of C3 as reported by many investigators [9,10]. According to our recent data, C5aR-AP has specific anti-C5a effects in rats and it suppresses the complement-mediated lethal shock [11].

In patients with glomerular injury induced by the antiglomerular basement membrane (GBM) antibodies, the preceding infection often induces or worsens the disease. Tomosugi and his coworkers reported that treatment of rats with subclinical dose of lipopolysaccharide (LPS) markedly enhanced the glomerular injury of heterologous phase of anti-GBM disease in rats and there was impressive thrombus formation in the glomeruli [12]. This model is considered as the human counterpart of infection-induced anti-GBM disease. In order to elucidate the role of complement, especially an anaphylatoxin C5a, in glomerular thrombus formation, we have investigated the effects of sCR1 and C5aR-AP in

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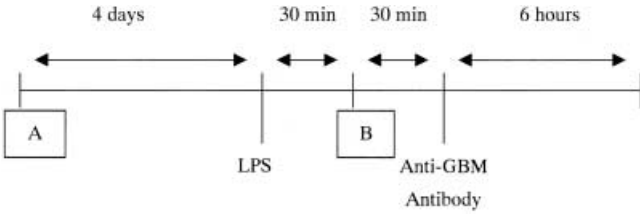
Table 1. Experimental protocol



Group (*n*)	(A) Pretreatment before injection of LPS*	(B) Treatment before injection of anti-GBM antibody†
I (12)	none	vehicle
II (6)	none	sCR1‡
III (9)	none	C5aRA §
IV (5)	Cyclophosphamide ¶	vehicle

*Intravenous injection of 2·5 μg/kg of LPS 1 h before the injection of anti-GBM antibody.
† Intravenous injection of 2 mg/rat of anti-GBM antibody.
‡ Intravenous injection of 20 mg/kg of sCR1 at 30 min before the injection of anti-GBM antibody.
§ Intravenous injection of 3 mg/kg of C5a receptor antagonistic peptide at 30 min before the injection of anti-GBM antibody.
¶ Intraperitoneal injection of 200 mg/kg of cyclophosphamide 4 days before the injection of anti-GBM antibody.

this model. The results obtained in the present work demonstrated that the glomerular thrombus formation was dependent on neutrophils and partially mediated by C5a but not MAC. The present study for the first time elucidated the role of anaphylatoxin C5a in the thrombus fromation in glomerulonephritis.

MATERIALS AND METHODS

Animals

Female Japanese white rabbits weighing about 2·5 kg and female Wistar rats weighing about 140 g were purchased from Chubu Kagaku Shizai (Nagoya, Japan) and allowed free access to food and water. In case of rats, blood samples were taken from tail vein and all reagents were intravenously administered to rats under ether anaesthesia. All experiments described here were carried out according to The Animal Experimentation Guide of Nagoya University School of Medicine.

Reagents

Rabbit antibody against rat GBM (RbAGBM) was prepared from rabbit antiserum to rat GBM as described previously [13]. Briefly, rabbits were intradermally immunized with rat GBM in Freund's complete adjubant every 2 weeks. One week after the third immunization, immune sera were obtained from the ear vein. The gamma globulin fraction was obtained by ammonium sulphate precipitation, and used as RbAGBM. RbAGBM was adjusted to 10 mg/ml in isotonic saline and stored at -70°C until use.

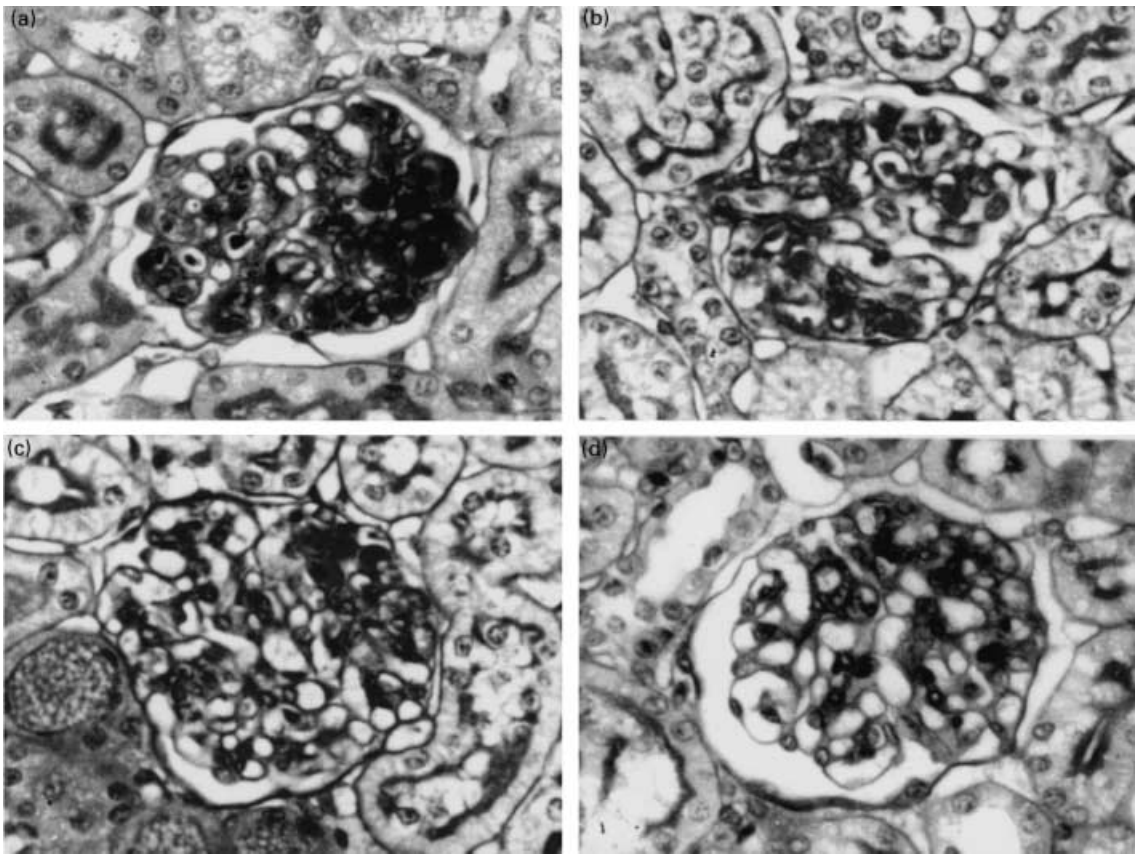


Fig. 1. Light microscopic appearance of the glomeruli. In Group I, most of the glomerular capillaries are occupied by the fresh thrombi. Thrombus formation in Group II and III is obviously less severe than that of Group I. In Group IV, thrombus formation is minimum. a, Group I; b, Group II; c, Group III; d, Group IV (PAS staining, × 400).

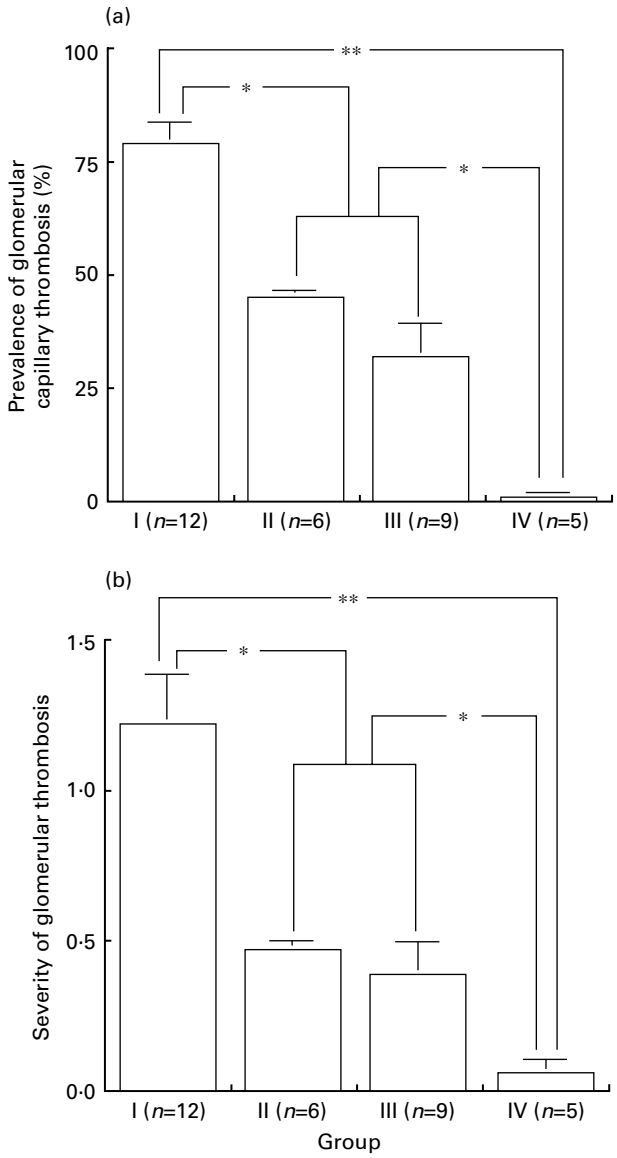


Fig. 2. Prevalence and severity of thrombus formation in the glomeruli. Both indices show that glomerular thrombus formation is significantly suppressed in Group II and III when compared to that in Group I. a, Prevalence of glomerular capillaries occupied by thrombi. b, Severity of glomerular thrombosis. (*$P < 0{\cdot}001$, **$P < 0{\cdot}0001$)

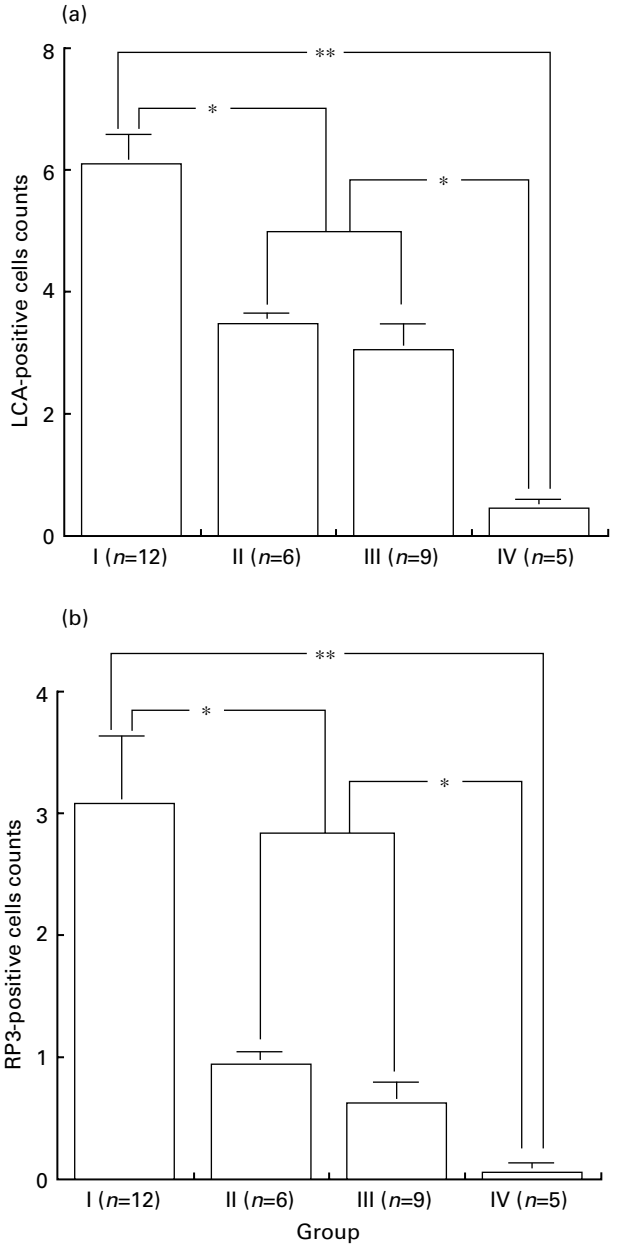


Fig. 3. The number of inflammatory cells infiltrated into the glomeruli. The data is given as the number of inflammatory cells per glomerular cross-section. a, LCA-positive cells; b, RP-3 positive cells (*$P < 0{\cdot}001$, **$P < 0{\cdot}0001$)

Table 2. Effects of C5a-R antagonist peptide on normal rats

	C5a-R antagonist ($n = 5$)	Vehicle ($n = 5$)
Leucocytes ($\times 10^6$/l)	6·6 ± 1·5	4·2 ± 3·3
Erythrocytes ($\times 10^{12}$/l)	6·648 ± 0·758	6·436 ± 0·071
Platelets ($\times 10^9$/l)	898 ± 47	734 ± 70
CH50 *(unit)*	60·6 ± 1·9	65·0 ± 0·3
Coagulation time (minutes)	4·5 ± 0·1	4·7 ± 0·2

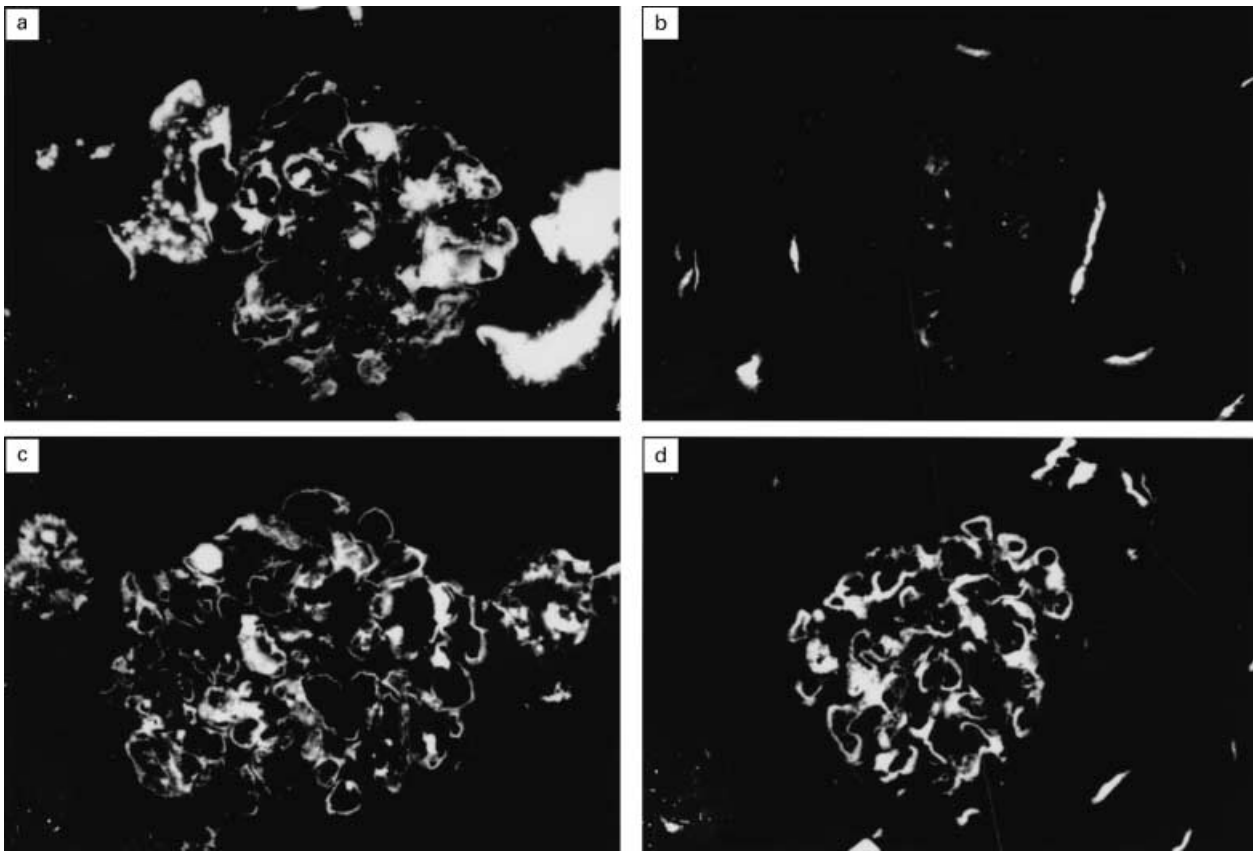


Fig. 4. Immunofluorescence pictures showing C3 deposition in the glomeruli. Deposition of C3 is similarly seen in Group I, III, and IV, while it is absent in Group II. a, Group I; b, Group II; c, Group III; d, Group IV $\times$ 400).

RbAGBM was negative for endotoxin when detected by Pregel-M detection kit (Seikagaku Kogyo Co. Ltd, Tokyo, Japan). Detection limit of Pregel-M was $<$ 50 pg/ml (or $<$ 0·25 EU/ml). Cyclophosphamide and LPS prepared from phenolic extracts of Salmonella typhimurium were obtained from Sigma Chemical Co. (St. Louis, MO). sCR1 was provided by AVANT Immunotherapeutics (Needham, MA) and Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). C5aR-AP, NmePhe-Lys-Pro-dCha-Trp-dArg [8], was originally reported as an antagonist of C5a receptor on human peripheral neutrophils. We have previously reported that this peptide had also specific inhibitory effects on rat neutrophils [11]. Intravenous administration of 3 mg/kg of this pepetide into rats completely rescued rats from the complement-dependent lethal shock [11]. Fluorescein isothiocyanate (FITC)-labelled goat antibody against rabbit IgG was purchased from Zymed Laboratories (San Francisco, CA). FITC-labelled rabbit antibody against rat C3 human fibrinogen were purchased from Cappel Lab. (Westchester, PA). FITC-labelled monoclonal antibody against rat leucocyte common antigen (LCA) (Clone OX1) was purchased from Dainippon Pharmaceutical Company (Osaka, Japan). RP3, a monoclonal antibody against rat neutrophils, was a gift from Dr F. Sendoh (Yamagata University, Yamagata, Japan) [14]. RP3 was labelled with FITC according to a method described by JL. Riggs *et al.* [15].

Effects of sCR1, C5aR-AP and cyclophosphamide on normal rats

Rats were intraperitoneally injected with either 20 mg/kg body weight of sCR1 or 3 mg/kg of C5a receptor antagonist peptide into normal rats. The dose used in this study was determined by our previous data [9–11]. Blood samples were obtained to study the peripheral blood count and serum complement activity 30 min after injection. Kidneys were also obtained for the histological study. In order to see the effects of cyclophosphamide, rats were intraperitoneally injected with 200 mg/kg body weight of cyclophosphamide. Rats were sacrificed 4 days later and their blood samples and kidney tissues were similarly examined.

Induction of thrombotic glomerulonephritis in rats

Rats were first intravenously injected with 2·5 μg of LPS in 0·5 ml of isotonic saline. One hour later, they were intravenously injected with 2 mg of RbAGBM to induce the disease. These rats were used as positive controls. Either one of LPS alone or RbAGBM alone did not induce any significant glomerular injury.

Experimetal protocol

In Group I, rats were injected with LPS and RbAGBM as described above. Thirty min before injection of RbAGBM, they were intravenously injected with 0·5 ml of saline (vehicle). In rats of Group II, 20 mg/kg body weight of sCR1 was intravenously injected 30 min before administration of RbAGBM. In rats of Group III, 3 mg/kg of C5aR-AP was similarly injected. In rats of Group IV, 200 mg/kg body weight of cyclophosphamide was intraperitoneally injected 4 days

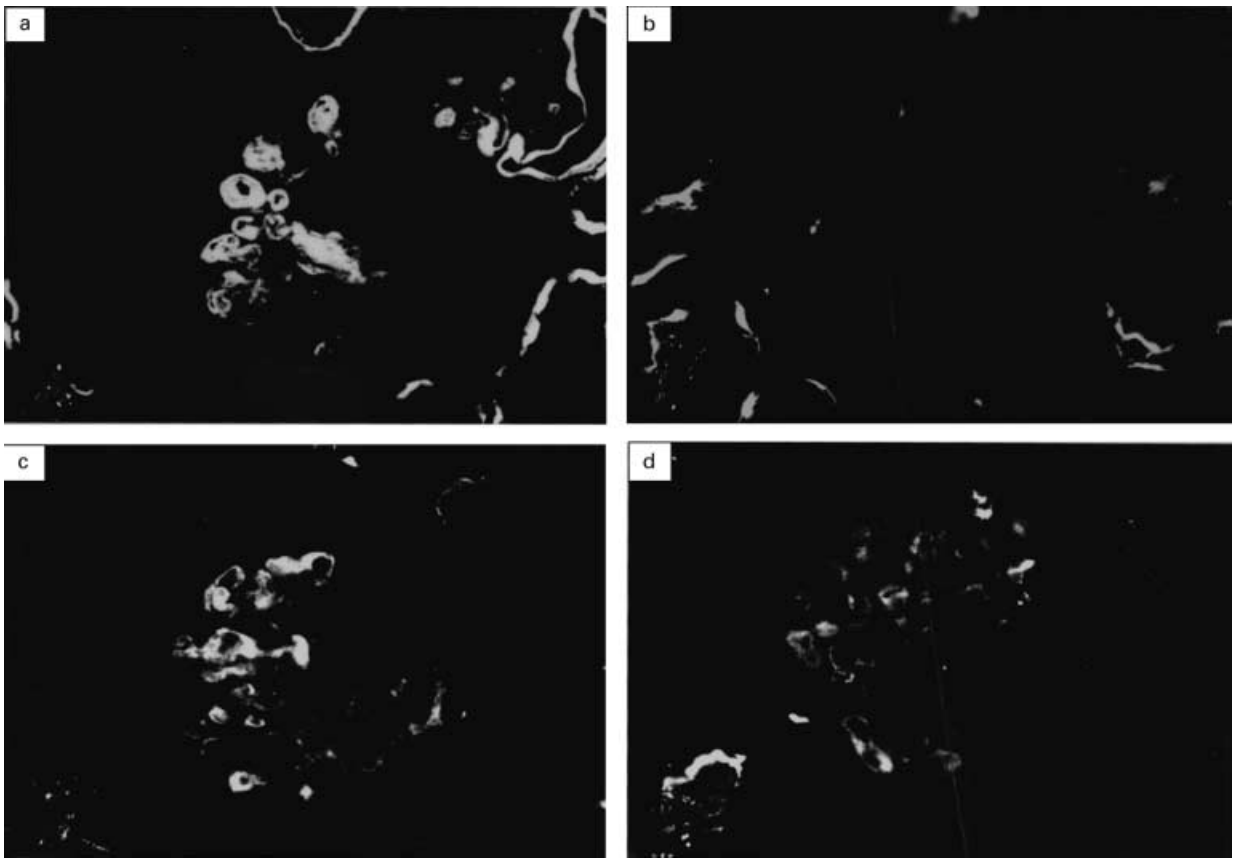


Fig. 5. Deposition of MAC in the glomeruli by FITC-labelled mAb against MAC. Segmental and weak deposition of MAC is observed in Group I, III and IV, while it is completely absent in Group II. a, Group I; b, Group II; c, Group III; d, Group IV $\times$ 400).

before induction of disease. The experimental protocol is summarized in Table 1. All rats were sacrificed 6 h after administration of RbAGBM, and the kidneys were obtained for histological and immunohistological examination. At the time of sacrifice, blood samples were also obtained.

Histological and immunohistological methods

For light miscroscopic examination, kidney specimens were fixed in methacarn fixative overnight and embedded in paraffin. Two μm thick sections were stained with periodic acid Schiff (PAS). To estimate the prevalence of glomerular thrombus formation, randomly selected 40 glomeruli were examined in each section. Glomerular thrombosis was semiquantified by the following formula;

Prevalence of Glomerular Capillary Thrombosis (%) = (total number of glomeruli with thrombus in at least one glomerular capillary $\div$ 40) $\times$ 100

To estimate the severity of thrombus formation in each glomerulus, the same glomeruli examined above were evaluated in a different way. Degree of glomerular thrombosis in each glomeulus was divided into 5 grades. 0; normal, 1; thrombus formation in less than 25% of total glomerular capillaries, 2; thrombus formation in 25–50%, 3; 50–75%, and 4: more than 75%.

Severity of Glomerular Thrombosis = (total sum of grade) $\div$ 40

For immunohistological evaluation, part of the kidney tissue was embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), snap frozen in liquid nitrogen and kept at $-$ 70°C until use. Two μm thick sections cut by a cryostat were fixed in acetone at room temperature for 10 min. They were then stained by FITC-labelled goat antirabbit IgG and rat C3.

For detection of infiltrating cells in the glomeruli, sections were stained with FITC-labelled rat LCA (Clone OX1). To detect the neutrophils in glomeruli, sections were incubated with FITC-labelled RP3. The number of positive cells were counted in randomly selected 40 glomeruli under epifluorescence microscopy (Olympus Optical Co. Ltd, Tokyo, Japan). The number of infiltrating leucocytes or neutrophils in a glomerulus was calculated according to the following formula;

Number of Leucocytes or Neutrophils in a Glomerulus = (total number of LCA- or RP3- positive cells in 40 glomeruli) $\div$ 40

Specimens for electron microscopic examination were fixed with glutaraldehyde, postfixed with osmium tetraoxide, and embedded in Epon 812 (Nisshin EM Co., Tokyo, Japan). Ultrathin sections were viewed with a H7100 electron microscope (Hitachi Co. Ltd, Ibaraki, Japan).

Measurement of serum creatinine level, serum complement activity, peripheral blood count and coagulation time

Serum creatinine level was measured using Cre-EN-KAINOS kit (Kainos, Co., Tokyo, Japan) according to a manufacturer's direction. For determination of serum complement activity, 50% complement haemolytic activity (CH50) was measured using a measuring kit containing sensitized sheep red blood cells (Ishizu Pharmaceutical Co., Osaka, Japan) according to a manufacturer's direction. One CH50 unit of complement activity lyses 50% of sensityzed sheep red blood cells whose concentration is adjusted to 5×10^8 in 7·5 ml of gelatin veronal buffer containing Ca++ (GVB++) for 90 min at 37°C. Total leucocytes and platelets were calculated by the automatic blood cell counter, STKS (Beckman Coulter Co., Tokyo, Japan). For measurement of whole blood coagulation time, blood samples were obtained from abdominal aorta. Coagulation time was measured by Lee-White's methods [16].

Statistical analysis

All values were provided as mean ± standard error. Statistical analysis was performed by one factor ANOVA for parametric data. When significant difference was present, analysis was further proceeded using Scheffe's *F*-test to detect the significant difference between any pair of 4 groups. For nonparametric data, statistical analysis was performed by K.ruskal-Wallis method. Correlation between serum creatinine and glomerular capillary thrombosis was analysed by Pearson's correlation coefficient. Significant difference was set when *P*-value was less than 0·05 (5%).

RESULTS

The effects of sCR1, C5aR-AP and cyclophosphamide on normal rats

Thirty minutes after intraperitoneal administration of sCR1, serum complement activity assessed by CH50 was below detection level. Conversely, C5aR-AP did not affect on the CH50 at all. sCR1 and C5aR-AP did not affect on the coagulation time nor the peripheral blood counts including RBC, leucocytes, and platelets. Rats treated with cyclophosphamide showed no significant abnormalities in their blood except for the marked reduction of leucocyte count (< 50 mm [3]). In all rats, histological examination of the kidney specimen revealed no morphological abnormality. These data are provided in Table 2.

The effects of sCR1 and C5aR-AP on the glomerular thrombus formation and leucocyte/neutrophil infiltration

In sCR1 treated rats (Group II) or C5aR-AP treated rats (Group III), significant reduction of glomerular thrombi was observed in both prevalence and severity criteria (Fig. 1 and 2). Similarly, Both sCR1 and C5aR-AP significantly inhibited the infiltration of leucocytes (Fig. 3a) and neutrophils (Fig. 3b) into the glomeruli. Neutrophil infiltration was more efficiently suppressed. These inhibitory effects were comparable in Group II and III rats.

Effects of sCR1 and C5aR-AP on the glomerular deposition of rabbit IgG, rat C3 and MAC

In rats of Group I (positive control), there was strong binding of C3 and MAC in the glomeruli (Fig. 4 a and 5 a). In rats treated with sCR1 (Group II), there was no deposition of C3 or MAC in the glomeruli (Figs 4b and 5b). In contrast, strong deposition of C3 and MAC was observed in C5aR-AP treated rats (Group III) and staining intensity was comparable to that seen in Group I rats (Figs 4c and 5c). In all groups of rats, glomerular deposition of rabbit IgG (RbAGBM) was strongly detected and there was no difference in the staining intensity among the experimental groups.

Effects of leukocyte/neutrophil deprivation

In rats deprived of leucocytes and neutrophils (Group IV), glomerular thrombus formation and leucocyte/neutrophil infiltration *were* almost completely inhibited (Figs 1,2 and 3). The intensity of glomerular deposition of C3 and MAC was comparable to those of Group I and III (Figs 4d and 5d).

DISCUSSION

In human, anti-GBM diseases are often triggered or worsened by the bacterial infection [17]. Glomerular thrombus formation is one of the characteristic pathological findings in such conditions. The rat model used in the present work therefore provides the useful tool to investigate this important pathological process. In anti-GBM diseases in humans or in experimental animals, strong binding of C3 and MAC is invariably observed along GBM. During the cascade of complement activation, various complement products are formed. Some of them are biologically active, and affects on the disease process. Among these, anaphylatoxins (C3a and C5a) and MAC are the substances whose biological effects have been well established in the various disease model [18–20]. In the present work, we examined first whether complement is involved in the development of thrombus formation. If complement is involved, we then tested whether the anaphyalatoxin, especially C5a, mediates the disease or not. From the present work, the following new findings were obtained.

First, complement is involved in the formation of glomerular thrombus formation in the present model of LPS-and anti-GBM-induced thrombotic glomerulonephritis. sCR1 potently inhibits complement activation at C3 level by inactivating C3 convertase [10]. The reason why we used sCR1 instead of cobra venom factor was explained as follows. Cobra venom factor strongly activates alternative pathway of complement, and the complement activating products are generated in large amount in the circulation [21]. When these byproducts are remaining in the circulation, we cannot properly evaluate the effects of complement. In contrast, sCR1 does not activate the complement system. Thus, sCR1 has advantage to cobra venom factor for evaluating influence of the complement system. It is reasonable that there is no deposition of C3 or MAC in the glomeruli in sCR1 treated animals.

Second, we found that C5aR-AP had the effects similar to sCR1 on the glomerular thrombus formation and leucocyte/neutrophil infiltration. It is interesting, however, that there was strong binding of C3 and MAC in this group, suggesting that MAC was not involved in the formation of glomerular thrombi. Both in sCR1 treated and C5aR-AP treated rats, there was significant reduction in the leucocytes and neutrophils infiltrating into glomeruli. These findings suggested that glomerular thrombus formation is dependent on leucocytes/neutrophils. Indeed in leucocyte/neutrophil depleted rats, there was complete inhibition of thrombus formation. Thus, thrombogenic effects of C5a seemed to be at least in part by its chemotactic effects for leucocytes/neutrophils [6,22].

In our previous study, C5aR-AP had specific inhibitory effects

on rat neutrophils to release myeloperoxidase when they are exposed to zymozan-activated serum [11]. This fact suggests that C5aR-AP has the stabilizing effects for neutrophils exposed to C5a. Furthermore, C5a has thrombogenic effects on the vascular endothelial cells, i.e. C5a up-regulates and procoagulant activities [5], and promote aggregation of pletelets [6]. Taken these information together, the potent anaphylatoxin, C5a, seems to play important roles in the glomerular thrombus formation in immune mediated glomerulonephritis.

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