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Progressive Glomerulonephritis with Increasing Proteinuria Induced by a Second Attack to the Mesangial Cell

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Summary. **We have previously reported that two consecutive injections of the anti-Thy 1.1 monoclonal antibody (mAb) 1-22-3 with an interval of two weeks causes irreversible mesangial alterations. However, the precise mechanism of the development of these alterations was not fully elucidated. In this study, we first investigated the prognosis of rats receiving the second intravenous injection of the mAb 1-22-3 at five weeks after the first injection when the amount of urinary protein excretion normalized. Two consecutive injections of mAb with an interval of five weeks also caused irreversible mesangial alterations with persistent proteinuria (155.8 ± 148.7 mg/day at six months). To analyze the mechanism of the development of these alteration, we then compared the acute phase alterations after the second injection (two injection group) with those after the first injection (single injection group). Although no difference in the staining intensity of bound mAb 1-22-3, rat C3 in glomeruli and serum CH50 levels at 30 min after the last anti-Thy 1.1 mAb injection was detected in either group, the number of neutrophils infiltrating into glomeruli 30 min after the first injection of anti-Thy 1.1 mAb was larger than that at 30 min after the second injection (8.7 ± 3.5 vs. 2.2 ± 0.8 / gcs, p = 0.0039). Additionally, the numbers of ED3 positive cells and CD8 positive cells infiltrating into glomeruli were significantly larger at five days after the second injection (two injection group) than after the first injection (single injection group). The results show that polymorphonuclear leukocyte (PMN) does not contribute to the progression of chronic glomerular lesions and that activated macrophages (ED3 positive cells) and CD8 positive cells are involved in the development and the progression of chronic glomerular lesions.**

Key words — progressive glomerular lesion, anti-Thy1.1 antibody, mesengial proliferative glomerulonephritis.

INTRODUCTION

Although chronic glomerulonephritis (CGN) is one of the most common diseases resulting in renal failure, its pathogenesis has yet to be fully understood. It is widely known that some patients of IgA nephropathy, the most common form of primary glomerulonephritis (GN) , do not show any progress, though others do develop to the end stage slowly or rapidly[1, 2]. Since some cases of both groups only show mild proteinuria and/or microhematuria without any severe clinical symptoms when they first come for treatment, it is sometimes difficult to distinguish those patients whose glomerular alterations will progress from those with a better prognosis in the early stage of the disease. Therefore, we have been trying to establish better experimental models of CGN to identify the differences in the initiation events in the early phase between cases with good and bad prognoses. We have previously reported that a single injection of the anti-Thy1.1 monoclonal antibody (mAb) 1-22-3 into rats causes severe but prominent mesangial alterations[3], and that two consecutive injections with an interval of two weeks causes irreversible mesangial alterations with persistent

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Abbreviations– CGN, chronic glomerulonephritis; GN, glomerulonephritis; mAb, monoclonal antibody.

proteinuria[4].

It is well known that the anti-Thy1.1 antibody binds its target antigen molecules on the mesangial cell surface and causes severe mesangiolysis and consequent mesangial proliferation[5]. In a previous study, we chose a period of two weeks after the first injection as a time point for the second injection because we had earlier observed that the target molecules of the antibody disappeared with mesangiolysis and just recovered to the normal range at two weeks after the antibody injection[4]. We confirmed that the second injection just after or one week after the first injection was not capable of inducing the irreversible mesangial alterations. The rat model of the irreversible mesangial alterations caused by two injections with an interval of two weeks is thus appropriate to analyze the mechanism of the development of CGN[6].

However, it has been claimed that the model is not suitable to compare the respective acute phase events after the first and the second injections. It has been pointed out that the alterations observed after the second injection are not only caused by the second injection but also a mixture with the remaining alterations caused by the first injection, because some infiltrating leukocytes into glomeruli can still be detected at two weeks after the first injection and some rats still show an abnormal range of proteinuria at this time point. Another item in question is whether the second injection at longer than two weeks after the first injection is capable of inducing the irreversible mesangial alterations. To address these problems, in the present study we investigated whether a second injection at five weeks after the first one is capable of inducing the irreversible mesangial alterations. We chose a period of five weeks after the first injection as a time point for the second because we had confirmed that no rats show abnormal proteinuria at five weeks after the first antibody injection and no abnormal range in the number of inflammatory cells in glomeruli was detected at this time point.

We demonstrate here that the second attack to the mesangial cell by the injection of anti-Thy1.1 mAb five weeks after the first attack causes irreversible mesangial alterations with persistent proteinuria. The proteinuria caused by the second injection is milder than that caused by the first injection, but increases with time. The model could be a mimic of human slowly progressive GN and serve as a novel model to analyze the mechanism of the development of CGN. It is conceivable that a study comparing the acute phase events caused by the second attack with those caused by the first attack will help to identify important factors in the initiation events resulting in progressive diseases.

MATERIALS AND METHODS

Animals

All experiments were performed using specific pathogen-free female Wistar rats weighing 150 to 170 g, purchased from Charles River Japan (Atsugi, Kanagawa). All animal experiments were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Preparation of mAb 1-22-3 has been described previously[3].

Experimental procedure (Experimental protocol is illustrated in Fig. 1)

Experiment 1

Five rats were intravenously injected with 1.0 ml saline containing 2 mg of mAb 1-22-3, and five other rats were injected with saline as a control. Twenty-four-hour urine samples were collected on days 1, 3, 5, 7, 10, and 2, 3, 4, and 5 weeks after the injection of saline or mAb 1-22-3, and all rats were sacrificed five weeks after the injection. Urine protein concentrations were determined by colorimetric assay (BioRad, Oakland, CA, USA) using bovine serum *albumin* (BSA) as a standard. After cardiac puncture for blood sampling, the kidneys were removed, weighed, cut into portions, and used for assessment by immunofluorescence (IF). To observe the cells infiltrating into glomeruli, the sections were incubated with mouse anti-rat OX-1 mAb (Serotec, Oxford, UK), which recognizes pan-leukocyte CD45 antigen, and then stained with FITC-conjugated anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA).

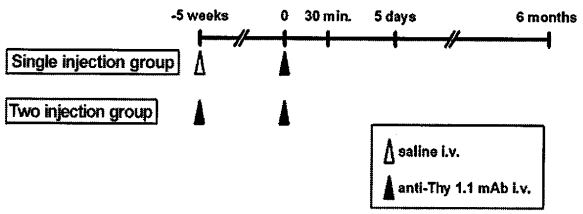

Fig. 1. Experimental protocol. Experimental procedure is illustrated. Female Wistar rats were intravenously injected with anti-Thy 1.1 mAb five weeks after saline injection (single mAb injection group) or an anti-Thy 1.1 mAb injection (two mAb injection group). Kidneys were removed just before and at 30 min, five days, and six months after the last injection.

Experiment 2

Ten rats were intravenously injected with 2 mg of mAb 1-22-3 twice with an interval of five weeks (two injection group). As a control (single injection group), 10 rats were injected with 2 mg of mAb 1-22-3 five weeks after the injection of saline. Twenty-four-hour urine samples were collected on days 1, 3, 5, 7, 10 and 14 after the injection of mAb, and then collected every week for six months. Urine protein concentrations were determined as above. All rats were sacrificed six months after the last injection. After cardiac puncture for blood sampling, the kidneys were removed, weighed, cut into portions, and used for an assessment by a light microscopy (LM).

Experiment 3

Five rats were intravenously injected with 2 mg of mAb 1-22-3 twice with an interval of five weeks (two injection group). As a control (single injection group), five rats were injected with 2 mg of mAb 1-22-3 for five weeks after the injection of saline. All rats were sacrificed 30 min after the last injection. After cardiac puncture for blood sampling, the kidneys were removed, weighed, cut into portions, and used for assessment by IF. Kidney sections were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Igs (Dakopatts, Glostrup, Denmark) to detect bound mAb 1-22-3. The sections were also stained with FITC-conjugated anti-rat C3 (Cappel, West Chester, PA, USA). To identify the inflammatory cells recruited into glomeruli, the sections were stained with RP-3, which recognizes polymorphonuclear leukocytes (PMNs), (kindly donated by Dr. Sendo, Yamagata University,Yamagata), and then stained with FITC-conjugated anti-mouse IgM (Southern Biotechnology Associates).

Experiment 4

Five rats were intravenously injected with 2 mg of mAb 1-22-3 twice with an interval of five weeks (two injection group). As a control (single injection group), five rats were injected with 2 mg of mAb 1-22-3 for five weeks after the injection of saline. All rats were sacrificed five days after the last injection. After cardiac puncture for blood sampling, the kidneys were removed, weighed, cut into portions, and used for assessment by IF. Kidney sections were stained with specific mAbs against rat lymphocytes or monocyte antigens. Mouse anti-rat mAb OX-38 (IgG2a, anti-CD4) was used as a helper T-cell marker, and OX-8 (IgG1, anti-CD8) as a cytotoxic/suppressor T-cell marker. OX-38 and OX-8 were precipitated from ascites using the corresponding hybridoma (European Collection of Animal Cells, Porton Down, Salisbury, UK). ED1 (IgG1, reactive with

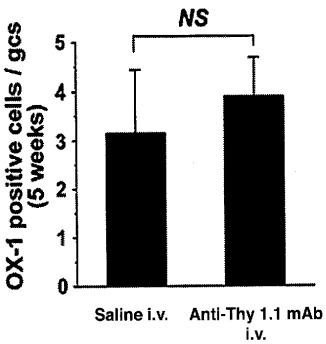

Fig. 2. OX-1 positive pan leukocytes in glomeruli five weeks after saline or mAb 1-22-3 injection. No difference in the number of OX-1 positive pan leukocytes in glomeruli is detected between the groups of saline and anti-Thy 1.1 mAb injection. The number of leukocytes in glomeruli recovered to normal ranges at five weeks after the anti-Thy 1.1 mAb injection.

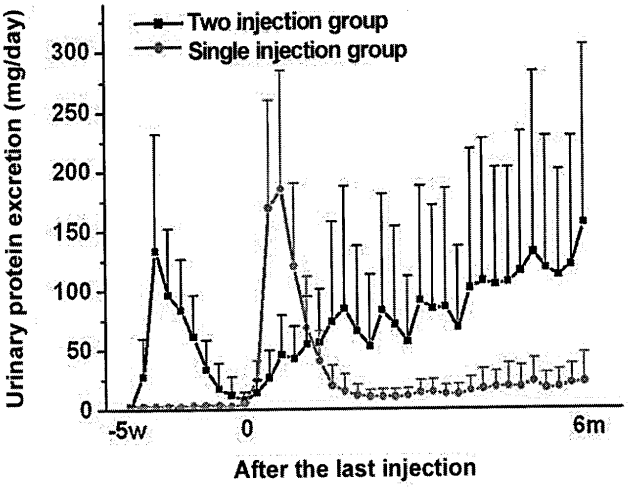

Fig. 3. Kinetics of proteinuria of rats after single or two injections of anti-Thy 1.1 mAb. A single intravenous injection of anti-Thy 1.1 mAb into Wistar rats caused massive but transient proteinuria, whereas two consecutive injections caused persistent proteinuria. Proteinuria increased gradually and persisted even six months after the second injection of anti-Thy 1.1 mAb.

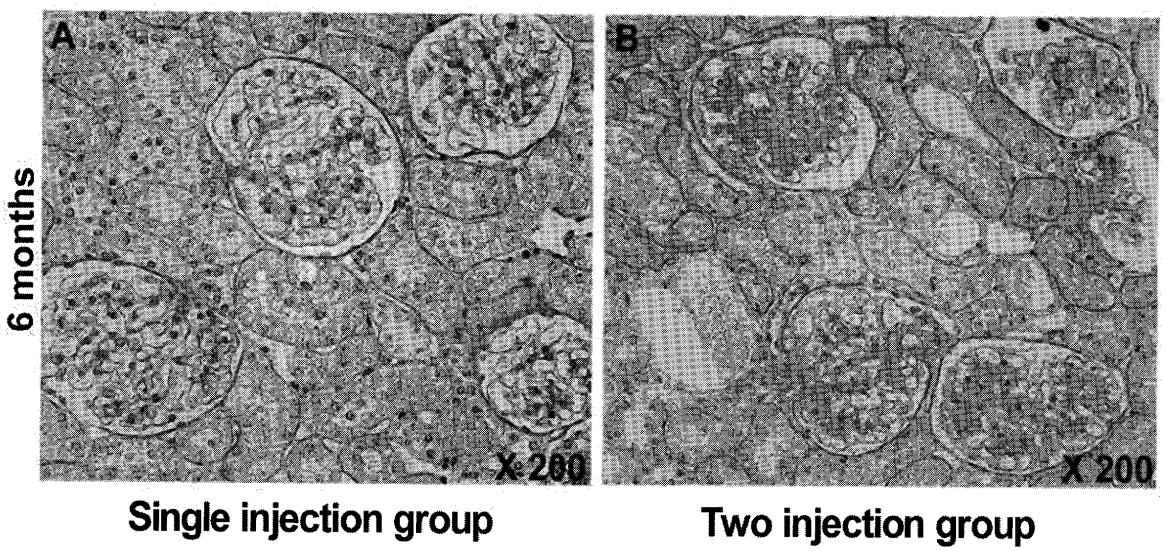

Fig. 4. Photomicrographs of periodic acid-Schiff (PAS) staining of kidney sections from rats six months after a single injection or two consecutive injections of anti-Thy 1.1 mAb 1-22-3. **A**. Almost normal glomeruli are observed at six months after a single injection of anti-Thy 1.1 mAb, **B**. while severe mesangial expansions are observed in most glomeruli at six months after two consecutive injections of anti-Thy 1.1 mAb.

pan monocytes/macrophages) and ED3 (IgG2a, reactive with macrophage sialoadhesin) were purchased from Chemicon International Inc. (Temecula, CA, USA). FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) and FITC-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates) were used as secondary antibodies. The number of mononuclear cells per glomerular cross section (c/gcs) was counted in 50 randomly selected full-sized glomeruli by an observer who was unaware of the experimental protocol.

Laboratory investigations

Serum creatinine, serum blood urea nitrogen (BUN) and urinary creatinine levels were measured. From the data on serum creatinine (Scr), urinary creatinine (Ucr), 24-hour urine volume (V), and body weight (BW) at sacrifice, the 24-hour endogenous creatinine clearance (Ccr) was calculated using the following formula:

$$\text{Ccr (ml/min/100 g BW)} = \text{Ucr (mg/dl)} \times \text{V (ml)} \times 1/\text{Scr (mg/dl)} \times 1/1440\ \text{(min)} \times 1/\text{BW (g)} \times 100$$

Light microscopy

For the LM study, a part of the kidney was fixed with Carnoy's solution, embedded in paraffin, cut into 4 μm sections, and stained with a periodic acid-Schiff (PAS) reagent.

IF

Tissue samples for IF studies were snap-frozen in pre-cooled n-hexane and stored at -70°C. Frozen sections 3-μm thick were cut with a cryostat and stained with antibodies as described above.

Statistical analysis

Statistical significance was evaluated using the unpaired t-test or the Mann-Whitney U-test. Values were expressed as the mean ± SD. Differences at $p < 0.05$ were considered significant. Data were analyzed using StatView for Windows (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Experiment 1

Leukocytes infiltrating into glomeruli five weeks after saline or mAb 1-22-3 injection were evaluated by counting the number of glomerular OX-1 positive cells. No difference in the number of OX-1 positive pan leukocytes in glomeruli was detected between the groups of saline and anti-Thy 1.1 mAb injection (Fig.

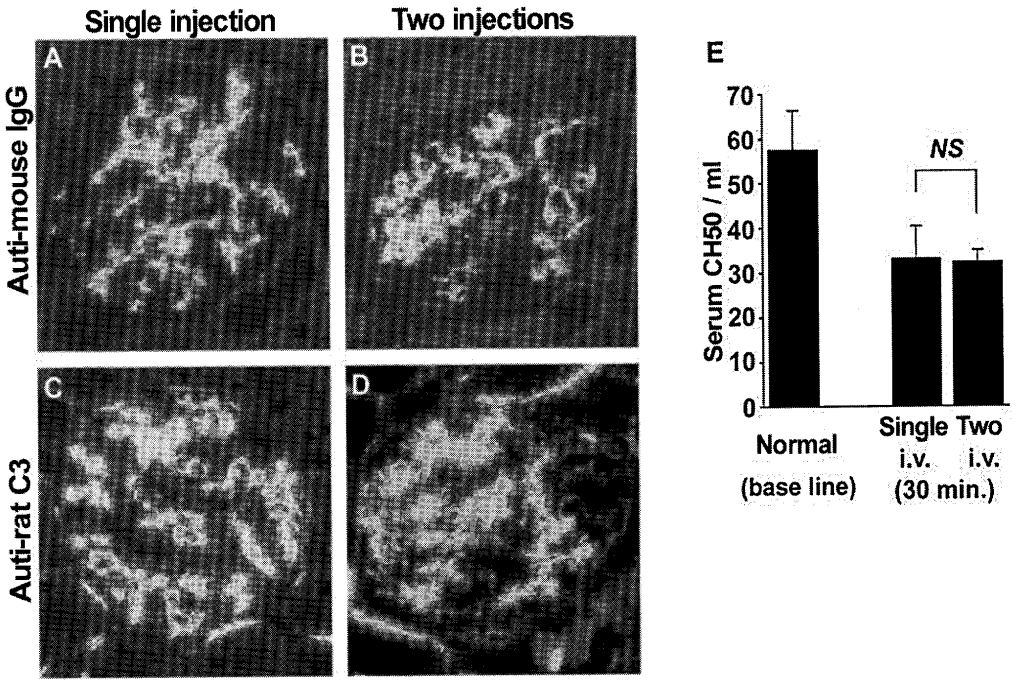

Fig. 5. Representative immunofluorescence (IF) findings of bound mAb 1-22-3 and C3 in glomeruli and the values of serum CH50. Representative IF stainings with FITC-conjugated anti-mouse Igs (**A** and **B**) and with FITC-conjugated anti-rat C3 (**C** and **D**) are shown. No difference in staining intensity of bound mAb 1-22-3 and rat C3 is observed between the groups of single and two anti-Thy 1.1 mAb injections. No difference in the value of serum CH50 was detected in either group (**E**).

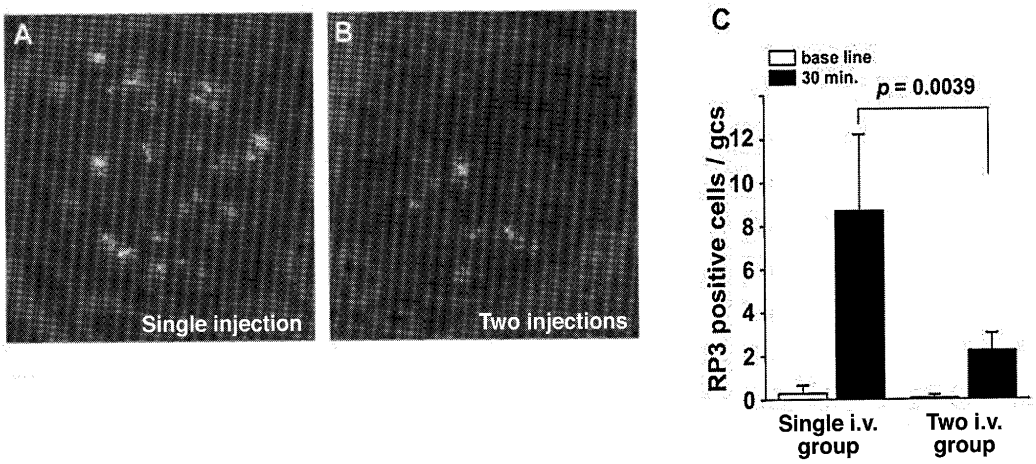

Fig. 6. The number of RP-3 positive cells infiltrating into glomeruli 30 min after the last injection of anti-Thy 1.1 mAb. **A**. Representative IF stainings of RP-3 positive cells in glomeruli after the first **B**. or the second **C**. injection of mAb 1-22-3 are shown. The number of RP3 positive cells infiltrating into glomeruli 30 min after the first injection of anti-Thy 1.1 mAb is larger than that of 30 min after the second injection.

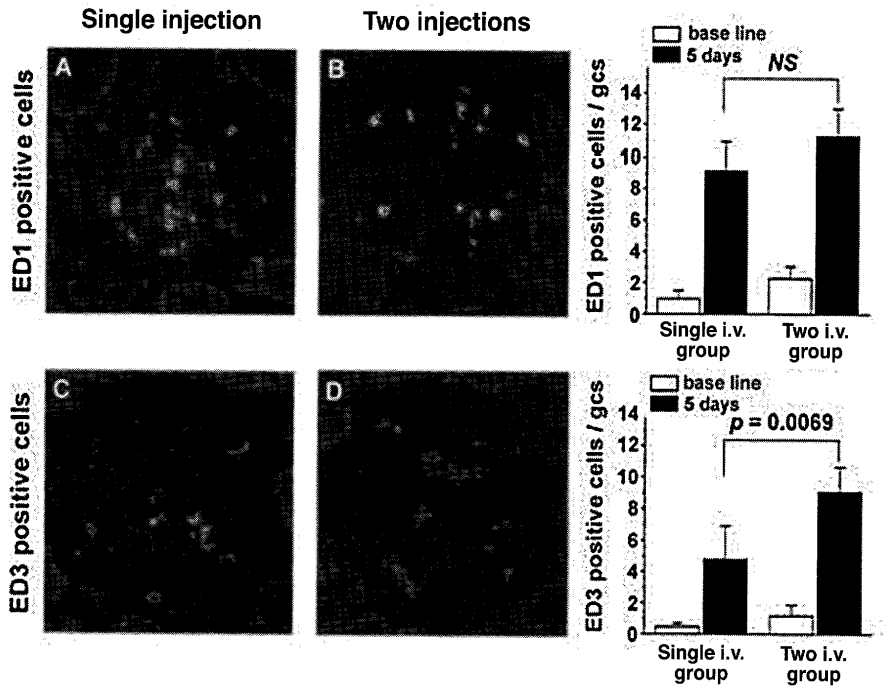

Fig. 7. The number of ED1 positive cells and ED3 positive cells infiltrating into glomeruli five days after the last injection of anti-Thy 1.1 mAb. Representative IF stainings of ED1 (**A** and **B**) and ED3 (**C** and **D**) are shown. There is no difference in the number of ED1 positive cells infiltrating into glomeruli five days after the last injection between the groups of single and two anti-Thy 1.1 mAb injections. The number of ED3 positive cells infiltrating into glomeruli five days after the second injection of anti-Thy 1.1 mAb (two injection group) is larger than that of five days after the first injection (single injection group).

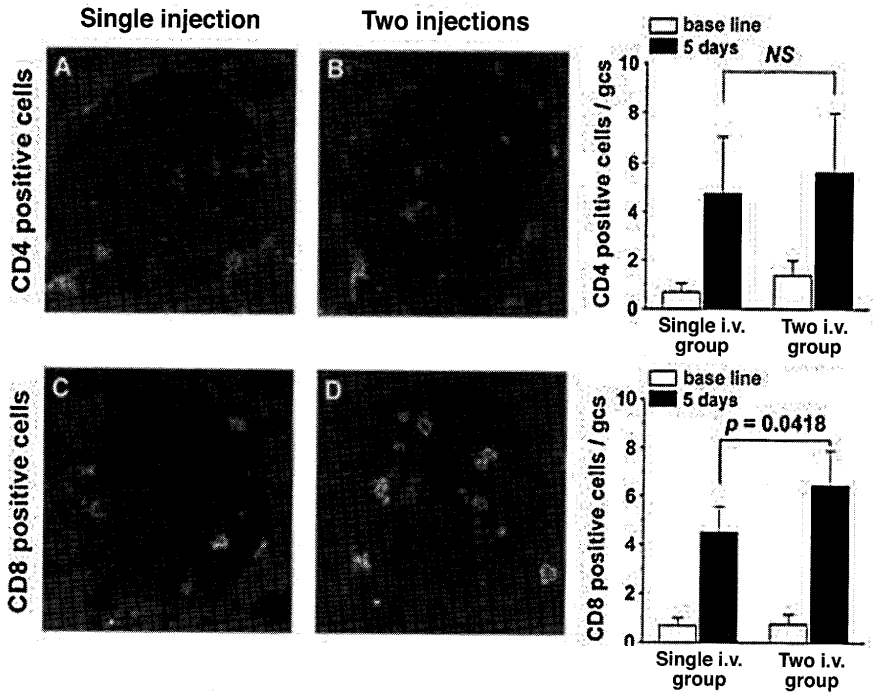

Fig. 8. The number of CD4 positive cells and CD8 positive cells infiltrating into glomeruli five days after the last injection of anti-Thy 1.1 mAb. Representative IF stainings of CD4 (**A** and **B**) and CD8 (**C** and **D**) are shown. There is no difference in the number of CD4 positive cells infiltrating into glomeruli five days after the last injection between the groups of single and two anti-Thy 1.1 mAb injections. The number of CD8 positive cells infiltrating into glomeruli five days after the second injection of anti-Thy 1.1 mAb (two injection group) is larger than that of five days after the first injection (single injection group).

2). The number of leukocytes in glomeruli recovered to normal ranges at five weeks after anti-Thy 1.1 mAb injection.

Experiment 2

A single intravenous injection of anti-Thy 1.1 mAb into Wistar rats immediately caused massive proteinuria, but this was transient. Proteinuria peaked on day 3, and then gradually decreased. The second injection caused persistent proteinuria. It gradually increased and persisted for more than six months after the last injection (Fig. 3). Almost normal glomeruli were observed at six months after a single injection of anti-Thy 1.1 mAb (Fig. 4A), while severe mesangial expansions were observed in most glomeruli at six months after two consecutive injections of anti-Thy 1.1 mAb (Fig. 4B). Two consecutive injections of anti-Thy 1.1 mAb induced mesangial lesions similar to human mesangial proliferative GN.

Experiment 3

No difference in staining intensity of bound mAb 1-22-3 (Fig. 5A and B) and rat C3 (Fig. 5C and D) was observed between the groups of single and two anti-Thy 1.1 mAb injections. No difference in the value of serum CH50 30 min after the last injection was detected in either group (Fig. 5E). The number of RP3 positive cells infiltrating into glomeruli 30 min after the first injection of anti-Thy 1.1 mAb was larger than that of 30 min after the second injection (8.70 ± 3.51 vs. 2.24 ± 0.81 / gcs, $p = 0.0039$, Fig. 6).

Experiment 4

There was no difference in the number of ED1 positive cells infiltrating into glomeruli five days after the last injection between the groups of single and two anti-Thy 1.1 mAb injections (9.14 ± 1.78 vs. 11.23 ± 1.80 / gcs, $p = 0.1025$, Fig. 7). The number of ED3 positive cells infiltrating into glomeruli five days after the second injection of anti-Thy 1.1 mAb (two injection group) was larger than that of five days after the the first injection (single injection group) (4.78 ± 2.11 vs. 9.06 ± 1.61 / gcs, $p = 0.0069$, Fig. 7). There was no difference in the number of CD4 positive cells infiltrating into glomeruli five days after the last injection between the groups of single and two anti-Thy 1.1 mAb injections (4.74 ± 2.26 vs. 5.61 ± 2.37 / gcs, $p = 0.5669$, Fig.8). The number of CD8 positive cells infiltrating into glomeruli five days after the second injection of anti-Thy 1.1 mAb (two injection group) was larger than that of five days after the first injection (single injection group) (4.49 ± 1.07 vs. 6.44 ± 1.45 / gcs, $p = 0.0418$, Fig. 8).

DISCUSSION

Thy 1.1 nephritis is characterized by severe mesangiolysis, the recruitment of inflammatory cells, and the consequent mesangial proliferation and matrix expansion[5]. Although it is widely used as a model of human mesangial proliferative GN[7, 8], the GN model induced by a single injection of anti-Thy 1.1 mAb is reversible, and mesangial alterations and proteinuria are normalized within one month after disease induction. Thus, the model is not suitable to investigate the progressive mechanism of mesangial proliferative GN. Our group has been trying to establish a better model with prolonged mesangial alterations with persistent proteinuria. We have already reported on two models: the first is caused by two consecutive injections of anti-Thy 1.1 mAb 1-22-3 with an interval of two weeks[4], and the second is by a single injection of anti-Thy 1.1 mAb to unilaterally nephrectomized rats[9].

In this study, we demonstrated that the second injection of anti-Thy1.1 antibody five weeks after the first injection caused irreversible mesangial alterations with increasing proteinuria. It should be emphasized that the second attack five weeks after the first, when the mesangial alterations, inflammatory reactions and proteinuria caused by the first attack were already recovered to normal ranges, was capable of inducing irreversible glomerular alterations. In this model, severe glomerular alteration with mesangial matrix expansion was still evident at six months after the second injection of anti-Thy1.1 mAb (Fig. 4). Proteinuria caused by the second attack with the antibody was milder than that caused by the first, but it increased with time, and reached the average value of 155.8 mg/day at six months after the second attack (Fig. 3). Although we detected a relatively large individual difference in the amount of proteinuria, we observed in the LM study that all rats showed severe mesangial alterations without remarkable individual differences. The model could be a mimic of human slowly progressive GN.

The present study clearly shows that, if glomeruli are once injured, even after their alteration has recovered and they appear normal, the glomeruli have a high susceptibility to the next attack. We precisely analyzed the early event just after the second attack (two injection group) and compared it with that after the first attack (single injection group). It is plausible that this study will help to identify critical factors resulting in irreversible glomerular alterations. We first confirmed that there are no differences in the staining intensity of bound mAb 1-22-3, rat C3 in glomeruli, and serum CH50 levels detected at 30 min after the injection between the two groups (Fig. 5). These results show

that the potency of the attacks against the mesangial cell is same for both groups.

Next, we analyzed the inflammatory responses in glomeruli after the second mAb injection, and compared them with those after the first injection. The current study shows that the number of PMN (RP3 positive cells) recruited into glomeruli in rats 30 min after the second injection (two injection group) was clearly less than after the first injection (single injection group) (Fig. 6). Although the roles of PMNs recruited into glomeruli are uncertain, the results obtained in this study show that PMNs do not contribute to the progression of chronic glomerular lesions.

We then analyzed the inflammatory cells recruited into glomeruli five days after injection since we observed in our previous study that the number of the glomerular infiltration cells peaked on days 5-7 after the injection of anti-Thy 1.1 mAb[3, 4]. Another reason why we chose this time point was that the proteinuria peaked on days 3-5 after the injection of anti-Thy 1.1 mAb. The results show that there is no significant difference in the number of pan-macrophages (ED1 positive cells) and CD4 positive cells were recruited in glomeruli between both groups (Fig. 7 and 8), while the numbers of ED3 positive cells and CD8 positive cells infiltrating into glomeruli are significantly larger at five days after the second injection (two injection group) than after the first injection (single injection group) (Fig. 7 and 8). These results suggest that activated macrophages (ED3 positive cells) and CD8 positive cells are involved in the development and the progression of chronic glomerular lesions.

In conclusion, the current study demonstrated that the second attack to the mesangial cell after the alterations caused by the first injection were normalized causes irreversible mesangial alterations with persistent proteinuria. The alterations could serve as a novel model to analyze the mechanism of the progression of CGN.

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