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Nephritogenic Properties of Nephrotoxic Guinea Pig Antibodies

I. Glomerulonephritis induced by Guinea Pig IgG_1 Antibody in Rats

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Summary. Guinea pig IgG_1 and IgG_2 were separated from guinea pig antisera against rat kidney using DEAE column chromatography with gradient elution. In vitro system, nephrotoxic IgG_1 antibody did not fix rat complement with rat kidney sediments, but did IgG_2. Nephrotoxic IgG_1 or IgG_2 was intravenously injected into rats respectively. Glomerulonephritis characterized by an immediate massive proteinuria was initiated by nephrotoxic IgG_1 antibody. Marked intraluminal fibrin formation and phagocytic accumulation of small numbers of neutrophiles and monocytes were observed in the glomeruli. Rat β1c globulin was only weakly seen along glomerular basement membrane of the biopsy specimens early in the development of nephritis. On the other hand, rats given a comparable dose of nephrotoxic IgG_2 antibody did not develop a significant proteinuria and glomerular changes despite localization of IgG_2 and rat β1c in the glomeruli. These observations suggest that non-complement mediated glomerular injury is present at least in the initial phase of Masugi nephritis.

In the past few years, two classes of 7S immunoglobulins (IgG_1 and IgG_2) have been described and characterized in the guinea pig. The IgG_1 and IgG_2 immunoglobulins have different electrophoretic mobilities (Benacerraf *et al.*, 1963) and different biologic properties: IgG_1 antibody has a capacity to sensitize guinea pig for passive cutaneous or systemic anaphylaxis (Ovary *et al.*, 1963), but is incapable of fixing complement and lysing red blood cells (Bloch *et al.*, 1963; Kourilsky *et al.*, 1963). IgG_2, on the other hand, has been known to fix complement, lyse cells and be capable of provoking Arthus reaction, but not passive anaphylactic reaction.

Meanwhile, as to the mechanisms of the initiation of Masugi nephritis, a sequence of immunobiological events has been noticed. Especially, there is a general agreement that participation of the complement-leucocyte system is essential for initiation of glomerulonephritis (Cochrane *et al.*, 1965; Cochrane, 1969). On the other hand, it is found that rats injected with duck nephrotoxic antibodies developed an immediate proteinuria, despite little or no fixation of C3 (Unanue and Dixon, 1964) and the absence of neutrophile accumulation (Cochrane, 1965). Some experiments are also reported that Masugi nephritis could be induced in complement deficient rabbits (Rother *et al.*, 1967) and complement deficient mice (Unanue *et al.*, 1967).

The present investigation was undertaken to study further the immunobiological properties of the initiation of Masugi nephritis, using nephrotoxic guinea pig IgG_1 antibody which is found to be incapable of fixing complement in the classical pathway. Nephritogenicity of nephrotoxic IgG_2 antibody is also described.

Materials and Methods

Animals. Wistar rats of either sex weighing 150 to 200 g were used.

Antigens. Renal antigen for immunization was prepared by the method described by Fujimoto *et al.* (1964). Renal cortices were obtained from perfused rat kidneys and ground in a Waring blendor. A 10% w/v suspension was prepared in saline. Soluble antigen of kidney was prepared by trypsinization of kidney sediment by the method of Cole (1951).

Preparation of Nephrotoxic Serum. Guinea pigs weighing 500 to 600 g were injected in footpads with 0.5 mgN of the antigen in an equal volume of incomplete Freund's adjuvant, followed by seven biweekly injections of the same antigen into the back muscle. They were bled 2 weeks after the last injection. Sera were absorbed with packed rat erythrocytes and a small quantity of rat serum until no hemagglutination and precipitin reactions were detected.

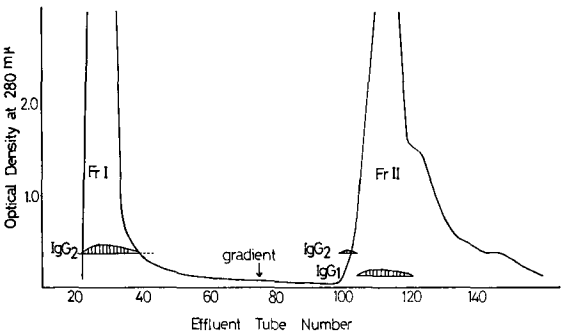

Fig. 1. A chromatographic pattern of 40% ammonium sulfate precipitate on DEAE cellulose column using gradient elution from 0.005 M, pH 8.5 to 0.59 M, pH 4.0 phosphoric acid-Tris buffers. IgG_1 fraction was mostly eluted in the second peak

Purification of IgG_1 and IgG_2 Fractions from Nephrotoxic Sera. Purification of immunoglobulin fractions was carried out according to the method described by Yagi *et al.* (1962) with diethylaminoethyl (DEAE) cellulose column chromatography. The serum was precipitated at 40% saturation of ammonium sulfate, and the precipitate was dissolved and dialyzed against 0.15 M saline and then a buffer consisting of 0.035 M Tris (hydroxymethyl) aminomethane (Tris) and 0.005 M phosphoric acid, pH 8.5. An aliquot of the sample was applied to a DEAE cellulose column which had been equilibrated with the same buffer. After the first peak was completely eluted with the buffer, the pH and salt gradient elution was started using the final buffer consisting of 0.5 M Tris and 0.59 M phosphoric acid, pH 4.0. IgG_2 was eluted in the first peak. The second peak contained most of IgG_1 (Fig. 1). When some contamination of IgG_2 was detected by immunoelectrophoresis, rechromatography of IgG_1 fraction was performed by the same procedure to devoid IgG_2. The preparation of IgG_1 fraction thus obtained was then passed through a Sephadex G 200 column (2.5 × 100 cm) to eliminate the possible contamination of IgM. The final preparation contained IgG_1 together with some α and β globulins.

Antisera. Anti-guinea pig IgG_1. Rabbit antibody specific for guinea pig IgG_1 was prepared by immunizing a rabbit with IgG_1 antibody specifically purified from hyperimmune guinea pig serum against hen's egg albumin (EA). Hyperimmune guinea pig serum was pooled and anti-EA was purified specifically by the method of Hong *et al.* (1965). The purified antibody containing guinea pig IgG_1 and IgG_2 was subjected to Starch block electrophoresis according to the method of Kunkel (1954). The fractions containing only IgG_1 were pooled, concentrated and used for the immunization of a rabbit. A rabbit received four injections of 400 μgN of this material emulsified in an equal volume of complete Freund's adjuvant with biweekly intervals and was bled 2 weeks after the last injection. This antiserum was absorbed with purified IgG_2 and was referred as anti-IgG_1.

Anti-Guinea Pig IgG_2. Normal IgG_2 for the immunization was prepared from normal guinea pig serum by the same chromatographic method as described above. Antiserum against IgG_2 was prepared by the same immunization procedure used for the preparation of anti-guinea pig IgG_1. The antiserum was absorbed with IgG_1 to render specific for IgG_2.

Anti-Rat β1c Globulin. The antiserum against rat β1c was prepared using zymosan according to the method described by Mardiney and Müller-Eberhard (1965).

Immunoelectrophoresis. Immunoelectrophoresis was carried out according to the method of Scheidegger (1955).

Quantitation of Kidney Fixing Antibody (KFAbs) in Nephrotoxic IgG_1 and IgG_2 Fractions in vivo. Radioiodination of IgG_1 and IgG_2 was carried out according to the method of Day *et al.* (1956). After extensive dialysis, the nitrogen content of the iodinated protein was estimated by nesslerization and its specific activity was determined. To calculate the rate of fixation of each nephrotoxic fraction to the kidneys and other organs, 150 μgN of I^{131} labeled antibodies were injected into groups of 3 rats. Estimation of the radioactivity in each organ was carried out by the method described by Bale and Spar (1954). As 90 per cent or more of nephrotoxic antibodies are fixed to GBM within 1 hour after intravenous injection (Unanue and Dixon, 1965), all rats were sacrified at 1 hour after the injection and organs were perfused with saline. Organs removed from the animals were blotted with filterpaper to remove excess fluids, and placed into test tubes which were then inserted into a well-type scintillation counter. The counts were corrected with background and decay of I^{131}. As a control, 100 μgN of I^{131} labeled normal guinea pig IgG_1 and IgG_2 were injected to groups of 3 rats and the degree of non-specific localization of immunoglobulins to various organs was estimated in a similar manner.

Complement Fixation Tests. Two-dimensional complement fixation tests were carried out by the method of Mayer (1961). Equal volumes (0.4 ml) of rat kidney sediments, nephrotoxic IgG_1 or IgG_2 antibody and 0.5 ml of fresh rat serum containing 5 CH_{50} units complement were incubated at 37° C for 1 hour. The mixture was centrifuged at 3000 rpm for 20 minutes, and then to the supernatant, 0.2 ml of EA containing 10^8 cells were added. After incubation at 37° C for 1 hour the degree of hemolysis was read macroscopically.

Induction of Glomerulonephritis by Nephrotoxic IgG_1 Antibody. Rats with urinary protein less than 1.0 mg/24 hr were used for the experiments. Nephritis was induced by a single intravenous injection of various amounts of IgG_1 or IgG_2 antibody. The amounts of urinary protein during 24 hr period were estimated according to the Kingsbury-Clark's method. In order to follow the dynamics of renal lesions, renal biopsy and autopsy were carried out at various times from 6 hr to 3 weeks after the injection. In control studies rats were given intravenously either 1 ml of normal guinea pig serum or 2 mgN of IgG_1 fraction of normal guinea pig serum.

Immunohistochemical Studies. Specimens obtained at renal biopsy and autopsy were snap frozen in liquid n-hexane at − 70° C. Frozen sections cut at 4 μ in a cryostat were stained with a fluorescent microscope (Type S-F, Nippon Kogaku). Conjugation of anti-rat β1c, anti-guinea pig IgG_1 and anti-guinea pig IgG_2 with Fluorescein isothiocyanate (FITC) was carried out according to the method of Wood *et al.* (1965). As controls of fixation of rat β1c in glomeruli, the blocking test was performed and normal rat kidney was stained with FITC-conjugated anti-rat β1c.

Histological Studies. For electron microscopy small blocks obtained at renal biopsy and autopsy were immersed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 to 2 hr at 4° C. The tissues were then post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer of pH 7.4 for 1.5 hr. After dehydration with graded ethanol, they were embedded in Epon 812. The ultrathin sections were cut on a Porter-Blum MT-1 microtome, doubly stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-8 or 11Ds electron microscope. Semithin sections (1 μ) were stained with PAS and Toluidine blue after removing the resin by the method by Imai *et al.* (1968) for light microscopic examination. Other tissue blocks were fixed in 10% formalin and embedded in paraffin for usual microscopic examination.

Results

Purity of IgG_1 Fraction. The purity of the final preparation of nephrotoxic IgG_1 fraction was tested by immunoelectrophoresis. As shown in Fig. 2, the

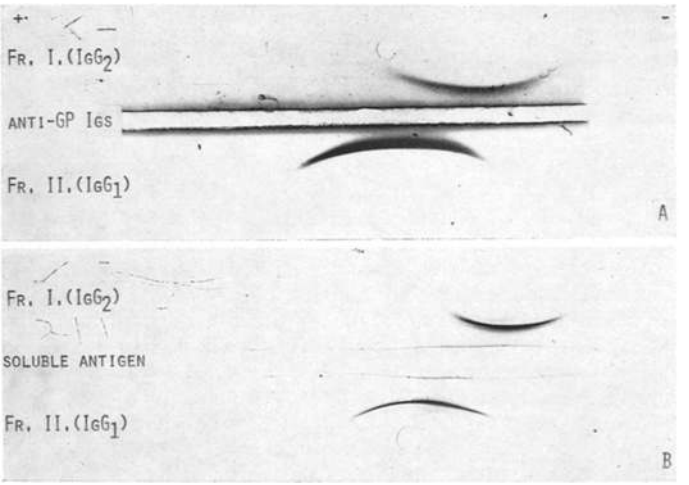

Fig. 2. Immunoelectrophoresis analyses of IgG_1 and IgG_2 fractions. 500 μgN of IgG_1 or IgG_2 fraction was applied into each well. IgG_1 fraction shows a precipitin band in the $\gamma 1$ region (1–a). This IgG_1 fraction developed with soluble kidney antigen forms a single distinct precipitin line corresponding to $\gamma 1$ region but not $\gamma 2$ (1–b)

Table 1. Localization of I^{131}-nephrotoxic IgG_1 and IgG_2 antibodies to various organs *in vivo*

Materials injected	Doses injected (μgN)	Per cent localized					
		Kidney	Heart	Lung	Liver	Spleen	Blood[a]
Nephrotoxic IgG_1 fr.[b]	150	2.536 ±0.511	0.078 ±0.017	0.569 ±0.104	9.303 ±1.297	0.830 ±0.091	2.714 ±0.073
Normal IgG_1fr.	100	1.625 ±0.354	0.044 ±0.014	0.171 ±0.112	5.721 ±1.228	0.190 ±0.019	3.385 ±0.145
Nephrotoxic IgG_2	150	2.338 ±0.150	0.093 ±0.012	2.003 ±1.227	13.202 ±2.988	0.743 ±0.135	1.958 ±0.139
Normal IgG_2	100	0.725 ±0.091	0.016 ±0.006	0.113 ±0.035	4.700 ±0.311	0.139 ±0.042	1.636 ±0.190

All results are average of 3 rats. Localization of each fraction to tissues was estimated at 1 hour after injection.

[a] Values are percent dose in 1 ml of blood.

[b] IgG_1 fraction.

fraction, 500 μgN of which were applied into a well, showed an only precipitin band in the $\gamma 1$ region with rabbit anti-guinea pig immunoglobulins in the immunoelectrophoresis. When the preparation was subjected to the immunoelectrophoresis with soluble antigen of trypsin digested kidney sediments, a precipitin band having a distinctly fast electrophoretic mobility developed. These results imply that the IgG_1 fraction contained mainly IgG_1 and the contamination of IgG_2 is negligible.

Table 2. Two-dimensional complement fixation tests with rat kidney sediments and nephrotoxic IgG_1 and IgG_2 antibodies

IgG_1 antibody system

IgG_1 (μgN)	Rat kidney sediments (μgN)						
	100	50	25	12.5	6.25	3.13	0
10	2[a]	3	4	4	4	4	4
5	2	4	4	4	4	4	4
2.5	2	4	4	4	4	4	4
1.25	2	4	4	4	4	4	4
0.63	2	4	4	4	4	4	4
0	2	4	4	4	4	4	4

IgG_2 antibody system

IgG_2 (μgN)	Rat kidney sediments (μgN)						
	100	50	25	12.5	6.25	3.13	0
10	0	0	1	1	2	2	4
5	0	1	2	3	4	4	4
2.5	1	2	3	3	4	4	4
1.25	1	3	3	4	4	4	4
0.63	2	3	4	4	4	4	4
0	2	4	4	4	4	4	4

Dotted line represents zone of complement fixation.
0 means no lysis, 4 means complete lysis.
[a] Anti-complementary effect.

Quantitation of Kidney Fixing Antibodies. The IgG_1 fraction was tested for its kidney fixing activity by the method described above. As shown in Table 1, 0.91 per cent of nephrotoxic IgG_1 fraction was fixed specifically to the kidney during 1 hr after the intravenous injection. On the other hand, 1.6 per cent of nephrotoxic IgG_2 was fixed to the kidney. It is worthy of note that 1.9 per cent of nephrotoxic IgG_2 which was over the dose of kidney fixing IgG_2 was fixed to the lung, while only 0.4 per cent of nephrotoxic IgG_1 was.

Complement Fixation Tests. Table 2 shows the results of two-dimentional complement fixation tests. In IgG_1 antibody system, IgG_1 antibody did not fix rat complement at all, whereas IgG_2 did in IgG_2 system.

Induction of Glomerulonephritis by Nephrotoxic IgG_1 Antibody. Table 3 represents the results of urinalysis in the rats with nephrotoxic IgG_1 fraction. All rats given more than 1 mgN of nephrotoxic IgG_1 containing 9.1 μgN or more of KFAbs developed an immediate proteinuria within 24 hr after the injection. Especially in rats injected with more than 2.7 mgN of nephrotoxic IgG_1, a massive proteinuria during the first 24 hr appeared. This proteinuria persisted for 2 to 3 months of the observation period. On the other hand, rats given less than 1.5 mgN of

Table 3. Induction of glomerulonephritis by nephrotoxic guinea pig IgG_1 or IgG_2 antibody

Rat No[a]	Nephrotoxic immunoglobulins	Total doses injected (mgN)	Amounts of kidney fixing antibodies (μgN)	Days after injection	Proteinuria (mg/day)	Immunofluorescent studies			
						GPIgG$_1$	GPIgG$_2$	Ratβ1c	RatIgG
801	IgG_1	13.3	121	1	140	3+[b]	—	1+	—
				14	256	3+	—	3+	3+
802	IgG_1	4.0	36.4	1	137	3+	—	1+	—
				died on 4	120	not done			
851	IgG_1	2.7	24.6	1	66	3+	—	1+	—
				14	210	3+	—	3+	3+
803	IgG_1	1.0	9.1	1	11.5	3+	—	1+	—
				14	5.0	3+	—	3+	3+
813	IgG_2	2.6	41.6	died at 20 min		not done			
814	IgG_2	1.9	30.6	died at 30 min		not done			
811	IgG_2	1.5	24.2	1	0.4	—	3+	3+	—
				9	0.1	—	3+	3+	3+
815	IgG_2	1.1	17.7	1	0.1	—	3+	3+	—
				14	0.6	—	3+	3+	3+

[a] These rats included in Table 3 are representative cases.
[b] Grade (3+) to (−) represents the intensity of fluorescene in glomeruli.

nephrotoxic IgG_2 containing 24.2 μgN of KFAbs survived but did not develop a significant proteinuria. Furthermore, if rats were given 30.6 μgN or more of KFAbs of IgG_2, they all died of the acute pulmonary edema within 1 hr after the injection as seen in rats given large amounts of nephrotoxic rabbit IgG (Shigematsu and Kobayashi, 1972).

Immunohistochemical Studies. In biopsy specimens obtained between 6 to 24 hr after the injection of nephrotoxic IgG_1, IgG_1 was observed to be localized along GBM in a linear pattern (Fig. 3), whereas IgG_2 was not detected. Rat β1c globulin was seen only weakly and usually locally along the same site in a linear pattern (Fig. 4). In the blocking test, rat β1c was negative in glomeruli of these biopsy specimens. In the survied group of rats given nephrotoxic IgG_2, IgG_2 together with β1c was distinctly stained despite no proteinuria and little histologic changes.

Histological Findings. As early as 6 hr after the injection, the glomerular endothelial linings detached themselves broadly from the basement membrane (Fig. 5).

At the same time small numbers of migrant neutrophiles and/or monocytes accumulated locally in the glomerular loops, particularly in their peripheral parts (Fig. 5, 8). Local fusions of epithelial foot processes were also seen. In the 24 hr biopsy specimen an accumulation of the materials with a staining property of fibrin was prominent in the glomeruli (Fig. 6). The fibrin mass often clung to the

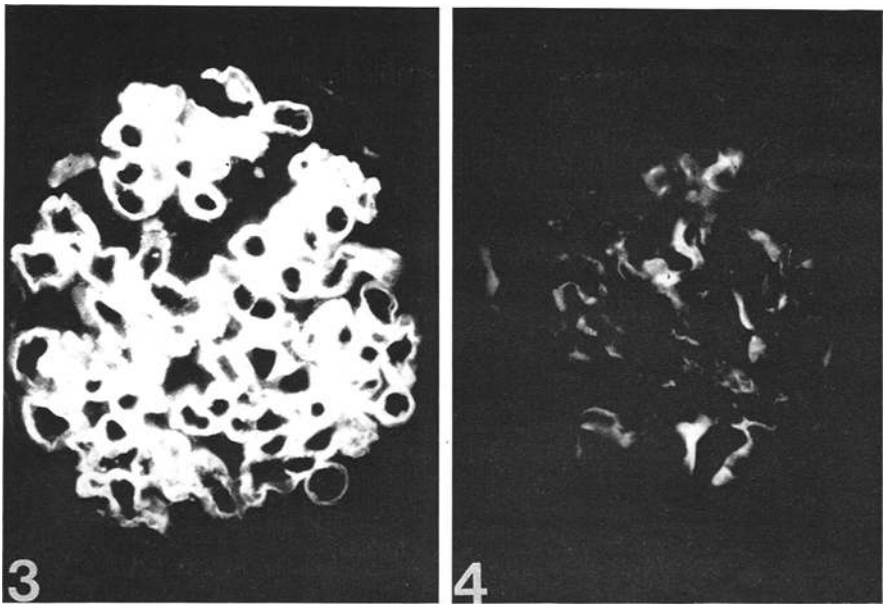

Fig. 3. Fluorescent micrograph of a glomerulus from the biopsy specimen obtained 24 hr after the injection of NTIgG$_1$. Guinea pig IgG$_1$ is brilliantly seen along GBM in a linear pattern

Fig. 4. Fluorescent micrograph of a glomerulus from the same specimen as that of Fig. 3 Rat β1c globulin is slightly seen along GBM

basement membrane and some capillary lumina were entirely occupied by these materials. In such areas the endothelial linings were usually absent or lifted up by the depositions of fibrinoid material (Fig. 9). Cell debris or osmiophilic granular materials were intermingled with the fibrin masses. The glomerular changes on the 2 to 3 day were similar to those found in the 24 hr-biopsy specimen, but, at this stage, infiltration of small numbers of monocytes or macrophages were noted in the glomeruli. Some glomerular loops were occluded by massive fibrin thrombi with an occasional partial necrosis of the glomerular tissues. Eleven to 14 days after the injection, the thrombotic changes of the glomeruli rather regressed, but the persistence of fibrinoid materials was still observed in the loops in varying degrees. The glomerular capillary wall showed local edematous thickening with the moderate increase of the mesangial matrix (Fig. 7).

Discussion

Present investigation demonstrated that glomerulonephritis characterized by massive formation of fibrin in the glomerular capillary lumen with minimum phagocytic reaction was induced by nephrotoxic guinea pig IgG$_1$ fraction.

It is reported that guinea pig IgG$_2$ antibody is capable of fixing complement and provoking passive Arthus reaction, whereas IgG$_1$ antibody is not (Bloch *et al.*, 1963). A question arises as to whether the glomerulonephritis induced by nephro-

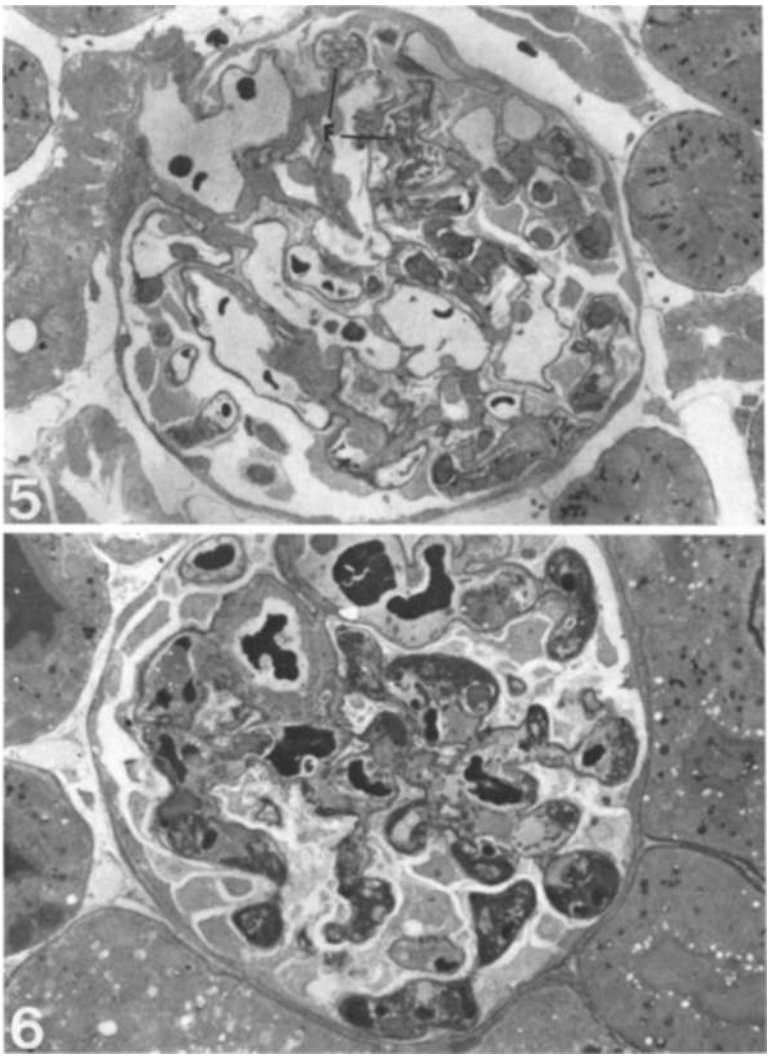

Fig. 5. 6 hr after the injection. Focal accumulation of neutrophiles in the peripheral capillary loops. Fibrin (*F*) is also observed in the capillary lumen. PAS and toluidine blue. 650 ×

Fig. 6. 24 hr after the injection. Massive intravascular fibrin formation is found in the peripheral part of the capillary loops. PAS and Toluidine blue. 650 ×

toxic IgG_1 fraction may be caused by IgG_2 antibody contaminating into IgG_1 fraction. However, the contamination of IgG_2 in IgG_1 preparation seemed to be little, if any: In the glomerulus of biopsy specimens of rats injected with nephrotoxic IgG_1 fraction, IgG_2 was not detected by fluorescent antibody methods. When a rat was injected with 1.0 mgN of nephrotoxic IgG_1 fraction containing 9.1 μgN of KFAbs, it developed an immediate proteinuria, while a rat injected with 1.5 mgN of nephrotoxic IgG_2 containing 24.2 μgN of KFAbs did not. These

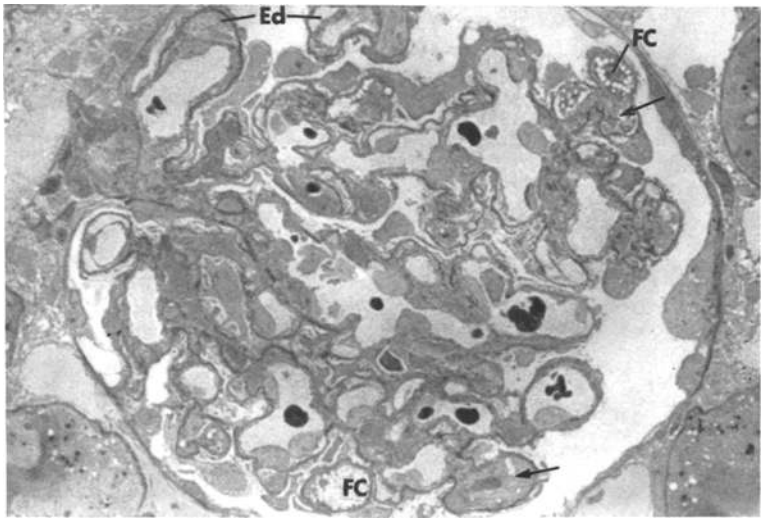

Fig. 7. 14 days after the injection. Intraluminal fibrinoid material is still present (arrows). Foam cells (*FC*) are observed in some capillary loops. The subendothelial space is seen to be edematous (Ed). PAS and toluidine blue. 650 ×

findings imply that nephrotoxic IgG_1 antibody are responsible for this glomerulonephritis.

If this is so, then, the next question is posed as to whether the complement system had a share in the initiation of the glomerulonephritis by IgG_1 antibody. It is unclear whether guinea pig IgG_1 antibody can not fix rat complement, although it is incapable of fixing guinea pig complement in the classical sequence of reactions (Bloch *et al.*, 1963; Keller *et al.*, 1968). Recently, Osler *et al.* reported that preformed immune aggregates composed of IgG_1 antibodies and antigens did interact with not only guinea pig, but also rat C3 and later components, despite little utilization of C1, C4 and C2, although IgG_1 antibody failed to activate the complement system in the conventional assays in which the order of addition was antibody, C and antigen (Osler, *et al.*, 1969; Sandberg *et al.*, 1970). However, the significance of the alternate pathway of complement fixation in the immunologic tissue injury is still unclear. In this experiment, nephrotoxie IgG_1 antibody did not fix rat complement with rat kidney sediments, whereas IgG_2 did. Moreover, in the initial glomerular changes induced by nephrotoxic IgG_1, rat β1c was only weakly seen along GBM, and an accumulation of only small numbers of neutrophiles and monocytes was observed, while the marked intraluminal fibrin deposition was the most dramatic feature. These are apparently different from the prominent phagocytic changes in rat Masugi nephritis initiated by nephrotoxic rabbit IgG antibody with an ability of fixing complement (Shigematsu, 1970). On the basis of these results, it is probable that the characteristic changes induced by nephrotoxic IgG_1 antibody is caused by non-complement mediated pathway at least in the classical sense of complement activation.

As mentioned above, the paucity of cellular reactions is another characteristic feature of IgG_1 nephritis. A small number of neutrophiles accumulated in the

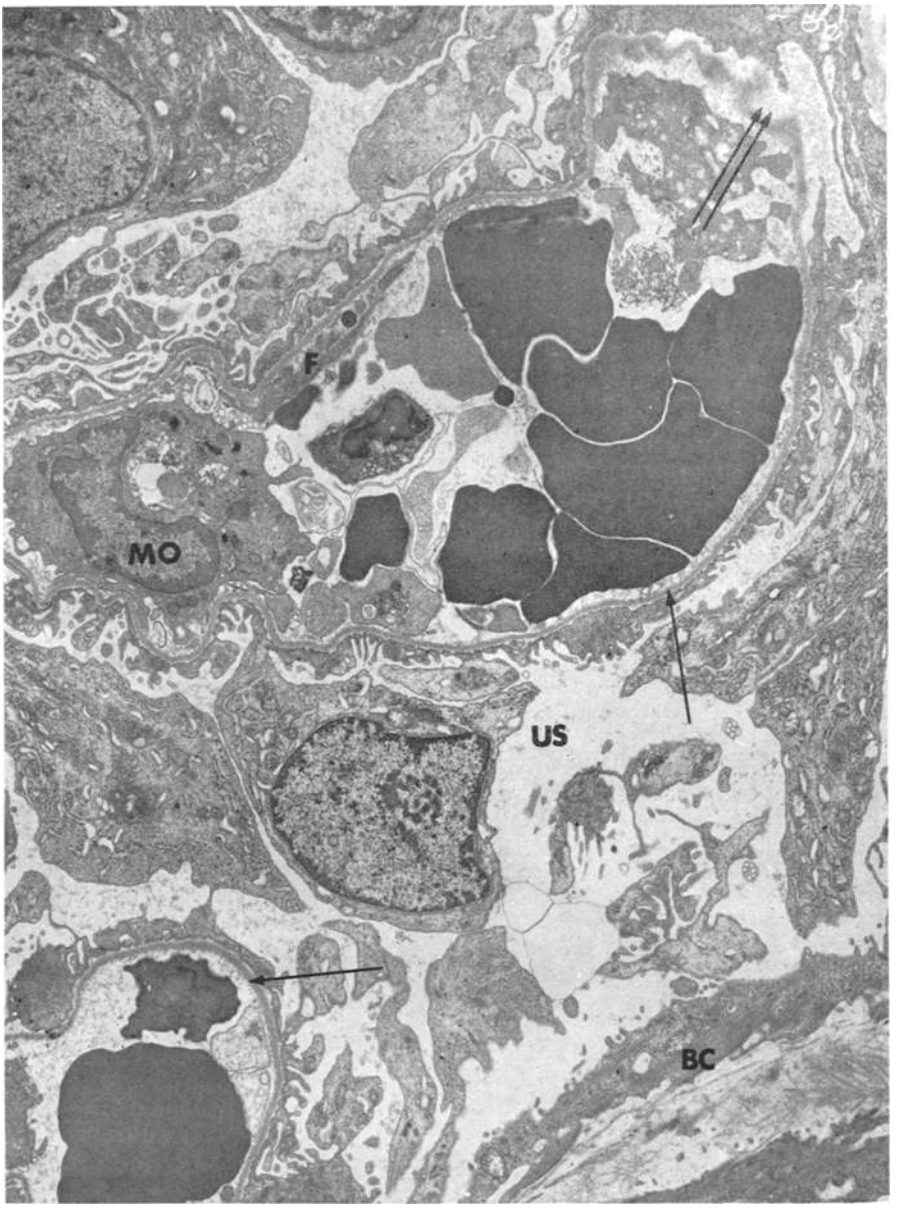

Fig. 8. 6 hr after the injection. Note broad exfoliation of endothelial lining from the glomerular basement membrane (arrow). Fibrin formation (*F*) and migrant monocyte (*MO*) are seen in the capillary lumen. Partial detachment of epithelial cytoplasm from the meandering basement membrane is found at double arrows. Urinary space (*US*) Bowmann's capsule (BC). 5200 ×

early stage of the nephritis disappeared almost completely at 24 hr after the injection. The number of mononuclear cells was also few which appeared instead of neutrophiles in the glomerulus. The deposition of fibrin, on the other hand, was

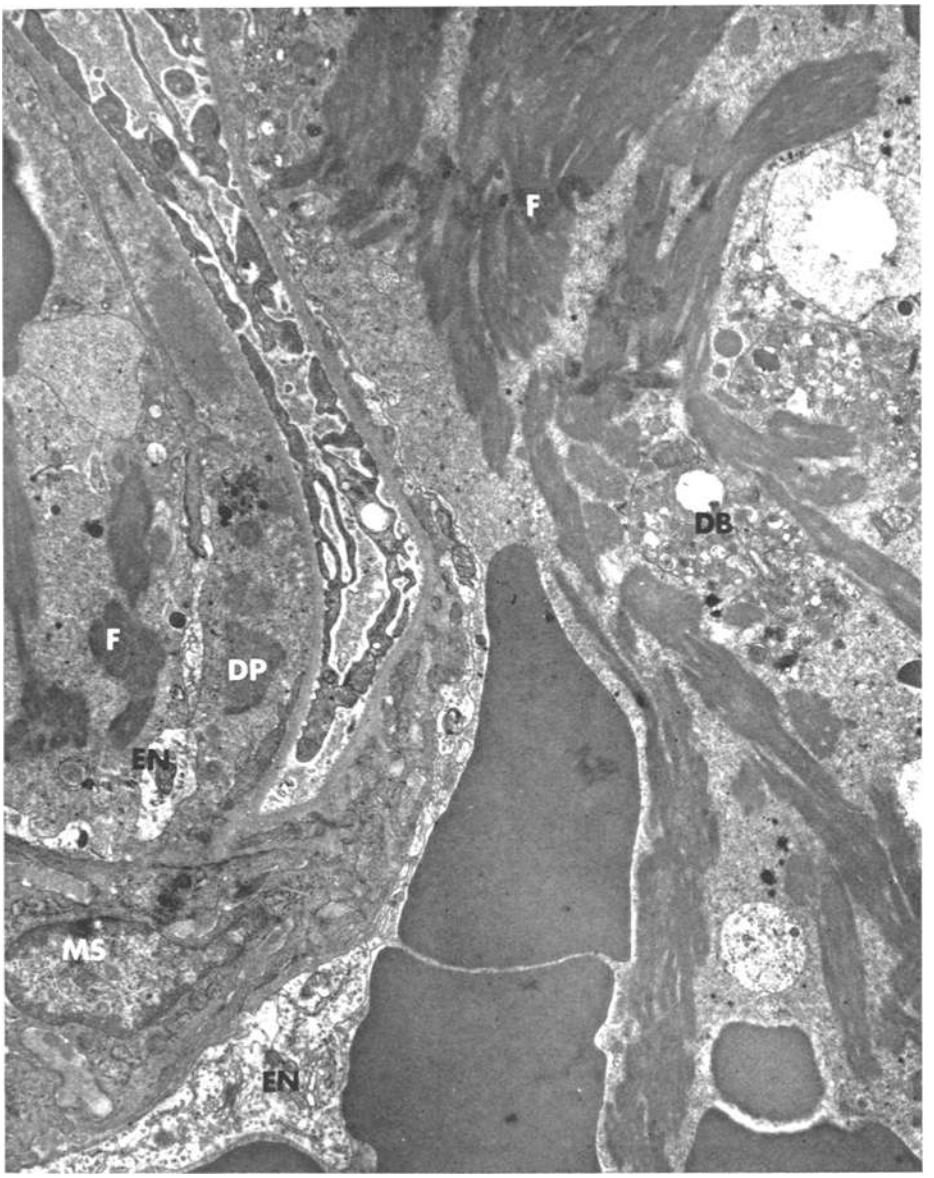

Fig. 9. 24 hr after the injection. Massive fibrin formation (F) in the capillary lumen. Deposits (*DP*) of fibrinoid material are seen beneath the endothelial cytoplasm (*EN*). Mesangial cells (MS). Cell debris (DB). 9600 ×

most striking in the glomerular capillary lumen, often clinging alongglomerular basement membrane. The formation of fibrin thrombi was found most evident in 2 to 3 day biopsy specimens and also seen to persist for 2 to 3 weeks after the injection. The fibrin deposition and/or thrombotic changes in the early phase of Masugi nephritis were particularly noticed by Vassalli *et al.* (1964) with respect

to its role in the development of glomerular changes. The acceleration of the blood coagulation might have been initiated by an early endothelial lesion caused by the nephrotoxic antibodies (Winemiller *et al.*, 1961) or local release of some blood coagulation activator as a consequence of the antigen-antibody interaction. The massive deposition of fibrin and the persistence of thrombotic changes in glomeruli observed in the present study may be partly due to the weakness of the cellular reaction of neutrophiles and monocytes which have phagocytic-reparative effects in the primary phase of rat Masugi nephrltis, as has been suggested by Shigematsu (1970).

On the other hand, nephrotoxic IgG_2 antibody which is capable of fixing complement had only a weak nephritogenicity at a comparable dose. Doses of over 30.6 μgN of KFAbs caused death from pulmonary complications. Guinea pig IgG_2 antibody in the preparation used may be lethal at nephrotoxic doses. It serves to confirm this possibility that the dose of lung fixing antibody in the IgG_2 preparation is much more than that of kidney fixing antibody. Although the reason why the kidney fixed IgG_2 antibody did not initiate complement mediated glomerular injury at a comparable dose is unexplained, it is suggested that non-complement mediated pathway may be of crucial importance in the initiation of at least some glomerular tissue injury.

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