Nephritogenic Properties of Nephrotoxic Guinea Pig Antibodies

II. Glomerular Lesions induced by $F(ab')_2$ Fragments of Nephrotoxic IgG₁ Antibody in Rats

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Summary. $F(ab')_2$ fragments were obtained from digestion of nephrotoxic guinea pig IgG₁ antibodies with pepsin. Development of an immediate but transient massive proteinuria was observed in rats injected with pepsin digested, divalent fragments. Although there was no demonstrable localization of rat $\beta 1c$ globulin in the early stage of glomerular lesions, nor accumulation of neutrophils in glomeruli, detachment of endothelial cells widely separated from the glomerular basement membrane (GBM) was observed at the site. As a control, $F(ab')_2$ fragments obtained from nephrotoxic rabbit IgG antibody with an ability of fixing complement were incapable of producing glomerular lesions as prominent as those obtained with nephrotoxic $F(ab')_2$ of IgG₁. It is suggested that the fixation of divalent fragments of nephrotoxic guinea pig IgG₁ antibody to GBM can cause damage of endothelial cells and a transient proteinuria without detectable participation of complement and neutrophils.

Guinea pig IgG₁ antibody is incapable of fixing complement at least in the classical pathway and capable of provoking passive anaphylactic reaction (Bloch *et al.*, 1963; Ovary *et al.*, 1963). It has also been known that a specific property of guinea pig IgG₁ antibodies which sensitize the skin of the guinea pig and rat for passive cutaneous anaphylaxis resides in its Fc portion (Lamm *et al.*, 1967; Nussenzweig *et al.*, 1966; Thorbecke *et al.*, 1963). Recently, however, Sandberg *et al.* (1971) reported that guinea pig IgG₁ antibody, when added in the form of preformed immune aggregates, initiated an attack on the complement sequence at C3, apparently bypassing C1, C4 and C2, and that this complement interaction site was located on the $F(ab')_2$ fragments.

Meanwhile, the disappearance of complement fixing activity of rabbit IgG antibodies by pepsin digestion was first observed by Taranta and Franklin (1961). Ishizaka *et al.* (1962) found that the structure essential for complement fixation was in the Fc portion of rabbit IgG globulin. Isliker *et al.* (1968) reported that the Clq binding site is located on the Fc portion of rabbit IgG molecule.

In our previous study (Kobayashi *et al.*, 1973), we were able to induce in rats with nephrotoxic guinea pig IgG₁ antibody a glomerulonephritis with the characteristics of an immediate massive proteinuria and marked intraluminal fibrin deposition, although without apparent participation of complement and phagocytes.

The present experiment was undertaken to determine to what structure of IgG₁ globulin the induction of glomerulonephritis by nephrotoxic IgG₁ is attrib-

uted. The glomerular lesions initiated by $F(ab')_2$ fragments of nephrotoxic IgG₁ antibody lacking Fc portion are described. As a control, the result obtained by pepsin treated divalent fragments of nephrotoxic rabbit IgG antibody which is capable of fixing complement is also discussed.

Material and Methods

Animals. Wistar rats weighing 150 to 200 g were used.

Antigens. Renal antigen for immunization was prepared by the method of Fujimoto *et al.* (1964). A 10% w/v suspension of rat renal cortices perfused and homogenized with a Waring blender was prepared in saline. Soluble antigen of kidney was prepared by the method of Cole *et al.* (1951).

Preparation of Nephrotoxic Serum. For the preparation of guinea pig nephrotoxic serum, guinea pigs weighing 500 to 600 g were immunized with the antigen by the same procedure already described (Kobayashi *et al.*, 1973). To obtain rabbit nephrotoxin, rabbits were injected at weekly intervals with 1 mgN of the renal antigen incorporated in an equal volume of incomplete Freund's adjuvant for 3 to 4 months, and were bled 2 weeks after the last injection. Each serum was absorbed with rat erythrocytes and serum until no hemagglutination and precipitin reactions were observed.

Purification of Guinea pig IgG₁ Globulin Fraction and Rabbit IgG Globulin. Purification of IgG₁ globulin fraction from nephrotoxic guinea pig serum was performed by the method of Yagi *et al.* (1962) using diethylaminoethyl (DEAE) cellulose column chromatography with the gradient elution. As previously described (Kobayashi *et al.*, 1973), IgG₁ globulin was eluted in the second peak with some α and β globulins but little IgG₂ was detected by immunoelectrophoresis. Rabbit IgG globulin was prepared by chromatography on a DEAE cellulose column eluting with 0.005 M phosphate buffer, pH 8.0.

Pepsin Digestion of Nephrotoxic Guinea pig IgG₁ Globulin Fraction and Rabbit IgG Globulin. Digestion of nephrotoxic guinea pig IgG₁ and rabbit IgG with pepsin was carried out according to the method described by Nisonoff (1964). Two per cent solution of IgG₁ globulin fraction or rabbit IgG was dialysed against cold 0.1 M acetate buffer, pH 4.0. Pepsin dissolved in the same buffer was added to the globulin solution at the ratio of 1 mg enzyme/100 mg protein. Digestion was allowed to proceed at 37°C for 18 hours. The reaction was stopped by adjusting pH to 8.0. The digestion products were dialysed against a borate buffered saline of pH 8.0, and passed through a Sephadex G 200 column to eliminate a possible contamination of undigested protein.

Antisera. Antisera were prepared as previously reported (Kobayashi *et al.*, 1973). Anti-guinea pig IgG₁ globulin was prepared by immunizing a rabbit with IgG₁ antibody specifically purified from hyperimmune guinea pig serum against hen's egg albumin. Anti-rabbit IgG globulin was prepared in a goat by repeated immunization with chromatographically purified normal rabbit IgG. Anti-rat β 1c globulin was prepared by using zymosan according to the method of Mardiney and Müller-Ehberhard (1965).

Immunodiffusion. Double diffusion in agar was performed by the method of Ouchterlony (1958).

Induction of Glomerular Lesions by $F(ab')_2$ Fragments of Nephrotoxic Guinea pig IgG₁ and Rabbit IgG. Wistar rats with urinary protein less than 1.0 mg/24 hr received a single intravenous injection of various amounts of $F(ab')_2$ of guinea pig IgG₁ or rabbit IgG. The amounts of urinary protein during 24 hr period were estimated according to the Kingsbury-Clark's method. Renal biopsies were carried out at various times from 6 hr to 3 weeks after the injection.

Immunohistochemical Studies. Specimens obtained by renal biopsy and at autopsy were snap frozen in liquid n-hexane at -70°C. Frozen sections cut at 4 μ in a cryostat were stained with each fluorescent antibody solution and examined under a Nikon fluorescent microscope (Type S—F, Nippon Kogaku). Conjugation of anti-guinea pig IgG₁, anti-rabbit IgG and anti-rat β 1c with Fluorescein isothiocyanate (FITC) was carried out by the method of Wood *et al.* (1965). The demonstration of $F(ab')_2$ of IgG₁ localized in tissues by fluorescent antibody methods were performed by using two kinds of antisera, FITC-conjugated anti-guinea pig

Fig. 1 A and B. Ouchterlony plate analyses of $F(ab')_2$ of guinea pig IgG₁. A. A precipitin band between untreated IgG₁ and anti-guinea pig IgG₁ unabsorbed with IgG₂ shows a spur against that of $F(ab')_2$. B. $F(ab')_2$ forms identical precipitin lines with untreated IgG₁ against soluble antigen

IgG₁ absorbed with IgG₂ and FITC-conjugated anti-guinea pig IgG₁ unabsorbed with IgG₂. If there is $F(ab')_2$ lacking the Fc portion of IgG₁ in tissues, fluorescence may be positive only by the latter conjugate. The demonstration of $F(ab')_2$ of rabbit IgG in tissues were done by the same technique using FITC-conjugated anti-rabbit IgG absorbed or unabsorbed with rabbit $F(ab')_2$.

Histological Studies. The procedures for light and electron microscopy were carried out according to the method described in the previous report (Kobayashi *et al.*, 1973).

Results

Purity of $F(ab')_2$ Fragments of Guinea pig IgG₁ or Rabbit IgG. The digested material was passed through a Sephadex G 200 column. The small amount of undigested protein eluted with the void volume was discarded, and the second peak was concentrated and used for the experiment. The purity of the final preparation was tested by immunodiffusion. As shown in Fig. 1, $F(ab')_2$ fragments of IgG₁ yielded reactions of partial identity with untreated IgG₁, when tested with unabsorbed rabbit anti-guinea pig IgG₁. Further, the preparation of the fragments showed precipitin lines identical with untreated IgG₁ against the soluble antigen

Table 1. Induction of glomerular lesions by pepsin digested $F(ab')_2$ fragments of nephrotoxic guinea pig IgG₁ antibody

Rat No.	Doses of $F(ab')_2$ injected (mgN)	Proteinuria (mg/day)					Immunofluorescent studies ^a			
							GBM		TEp	
		Day 1	3	5	7	14	GP Fab	rat $\beta 1c$	GP Fab	rat $\beta 1c$
804	3.5	140	90	50	8	2	+	—	+	—
806	1.0	150	10	5	(died)		+	—	+	—
861	0.3	15	4	3	5	2	+	—	+	—

^a The studies represent the result in the early stage of the lesions. GBM=glomerular basement membrane, TEp=proximal tubular epithelium, GP Fab=Fab fragment of guinea pig IgG₁.

Table 2. Induction of glomerular lesions by pepsin digested $F(ab')_2$ fragments of nephrotoxic rabbit IgG antibody

Rat No.	Doses of $F(ab')_2$ injected (mgN)	Proteinuria (mg/day)				Immunofluorescent studies ^a			
						GBM		TEp	
		Day 1	3	7	14	R Fab	rat $\beta 1c$	R Fab	rat $\beta 1c$
F 1	1.5	2.4	4.0	4.0	8.0	+	—	+	—
F 2	3.5	2.7	4.5	2.0	3.0	+	—	+	—
F 3	4.5	6.6	3.5	1.2	1.4	+	—	+	—
F 4	7.0	9.6	5.5	4.8	4.0	+	—	+	—
F 5	10.5	17.5	4.1	5.0	6.0	+	—	+	—
R 1 ^b	2.0 (NTRIGG)	20.0	8.0	5.0	10.0	+	+	—	—
R 2	4.0 (NTRIGG)	56.4	48.0	30.5	62.0	+	+	—	—

^a The studies represent the results in the early stage of the lesions. GBM=glomerular basement membrane, TEp=proximal tubular epithelium, R Fab=Fab fragment of rabbit IgG.

^b Two rats were injected with 2.0 or 4.0 mgN of untreated nephrotoxic rabbit IgG as a control.

of rat kidney (Fig. 1B). These findings indicate that the preparation of pepsin digested fragments has a precipitating efficiency with the same specificity. The purity of pepsin digested fragments of rabbit IgG was the same as $F(ab')_2$ of guinea pig IgG₁.

Induction of Glomerular Lesions by $F(ab')_2$ Fragments of Nephrotoxic Guinea pig IgG₁ and Rabbit IgG. Various amounts of $F(ab')_2$ fragments of nephrotoxic IgG₁ were injected into three rats. The results of urinalysis are displayed in Table 1. Rats given 3.5 mgN and 1 mgN of $F(ab')_2$ fragments which are equivalent to 10.5 mgN and 3 mgN of undigested IgG₁ globulin fraction with respect to the antigen-binding sites, developed a massive proteinuria within 24 hr after the injection. In contrast to the group receiving undigested nephrotoxic IgG₁ (Kobayashi *et al.*, 1973), however, the proteinuria was transient, lasting only for 3 to 5 days. The $F(ab')_2$ fragments of nephrotoxic rabbit IgG were injected into five rats. The results of urinalysis are shown in Table 2. Only the rat injected with 10.5 mgN of $F(ab')_2$ fragments developed a mild proteinuria within 24 hr, equivalent to 15 mgN of undigested rabbit IgG with respect to antigen-binding sites. This dose is about 8 times that of rabbit IgG necessary to induce a similar mild renal injury.

Fig. 2. Fluorescent micrograph of the glomerulus from a biopsy specimen obtained 6 hr after the injection of $F(ab')_2$ of guinea pig IgG₁. Guinea pig Fab is brightly seen along GBM in a linear pattern. Fluorescence is also observed in proximal tubular epithelium

Fig. 3. Fluorescent micrograph of the glomerulus from the same specimen as Fig. 2. No localization of rat $\beta 1c$ is seen in the glomerulus

Immunohistochemical Studies. In biopsy specimens obtained between 6 to 24 hr after the injection of $F(ab')_2$ fragments of nephrotoxic guinea pig IgG₁, no rat $\beta 1c$ globulin was observed along GBM (Fig. 3). $F(ab')_2$ of guinea pig IgG₁ were apparently localized not only along GBM in a linear pattern, but also in proximal tubule (Fig. 2). Fluorescence in the proximal tubule in biopsy specimens disappeared at 24 hr after the injection. Fluorescence of $F(ab')_2$ along GBM became much weaker at seven days after the injection. The fluorescence almost disappeared in 2 to 3 weeks. Likewise, in the rats given $F(ab')_2$ of nephrotoxic rabbit IgG, no rat $\beta 1c$ globulin was detected along GBM. $F(ab')_2$ of rabbit IgG showed the same pattern of distribution in GBM and proximal tubular epithelium as that of guinea pig IgG₁. The fluorescence of $F(ab')_2$ of rabbit IgG also disappeared from GBM within 3 weeks after the injection.

Histological Studies. In the rats injected with $F(ab')_2$ of nephrotoxic IgG₁, no prominent glomerular changes were found throughout the course of the experiment by light microscopy. Ultrastructurally, of note was that the endothelial linings exfoliated broadly from the glomerular basement membrane as early as 6 hr after the injection, particularly in the peripheral parts of the glomerular loops (Fig. 4). The junctional complexes between the adjacent endothelial cells were seen to be intact (Fig. 5). The lamina densa of GBM was loosened in some

Fig. 4. Glomerulus 6 hr after the injection of $F(ab')_2$ of guinea pig IgG₁. Detachment of the endothelial lining from the glomerular basement membrane is apparent (arrows). 4000 $\times$

degree in its endothelial side. It was also revealed that foot processes of the epithelial cells were in many parts flattened and fused. The lamina densa showed a meandering in these areas. At 24 hr, besides these changes of the glomerulus, localized aggregation of platelets was observed mostly within the widened spaces between the endothelial linings and basement membrane (Fig. 6). Small numbers of migrant monocytes were also seen in the lumen. In 2 to 3 days following the injection the basement membrane seemed to recover with nearly normal endothelial covering. However, the fusions of the epithelial foot processes were still

Fig. 5. Glomerulus 6 hr after the injection of $F(ab')_2$ of guinea pig IgG₁ (as Fig. 4). The junctional complexes (arrows) between the adjacent endothelial cells are intact. The endothelial side of the lamina densa is loosened. *PI* (platelet). $\times 16000$

Fig. 6. 24 hr after the injection. Platelets (*PI*) aggregate in the subendothelial space. The foot processes of the epithelial cells are flattened and fused. $\times 11000$

observed. In contrast, the glomeruli showed no remarkable changes in the rats injected with $F(ab')_2$ fragments of nephrotoxic rabbit IgG even with the electron microscope. Only a local detachment of endothelial cytoplasm from GBM was found in one rat which received 10.5 mgN of $F(ab')_2$ of rabbit IgG.

Discussion

The observation described in the present paper indicated a difference in the nephritogenicity between two kinds of $F(ab')_2$ fragments of nephrotoxic antibodies, guinea pig IgG₁ and rabbit IgG. The former fragments produced an immediate massive proteinuria but the latter did not at a comparable dose.

In some earlier studies, rats injected with large amounts of pepsin digested nephrotoxic rabbit IgG developed only a mild transient or no proteinuria (Small and Baxter, 1965; Fujimoto *et al.*, 1964). In order to explain the mild proteinuria, it was thought that pepsin digested divalent fragments of rabbit IgG fixed small amounts of complement (Schur and Becker, 1963). Recently, Reid (1971) found that $F(ab')_2$ fragments of rabbit IgG antibody could fix 30 to 50 per cent of guinea pig complement when they were aggregated with the appropriate antigen, whereas the fragments had no significant fixation of the C1 and C2 components. He suggested that $F(ab')_2$ of rabbit IgG antibody might fix complement by an alternate pathway which utilizes only the late components of C3 to C9.

In the present experiment, however, no specific localization of rat $\beta 1c$ globulin along GBM of rats given $F(ab')_2$ of nephrotoxic rabbit IgG and no histological changes in glomeruli were observed, and only a mild proteinuria developed with amounts of the fragment equivalent to about 8 times the dose of nephrotoxic rabbit IgG that was required to induce a similar proteinuria.

On the other hand, $F(ab')_2$ fragments of nephrotoxic guinea pig IgG₁ initiated an immediate massive proteinuria with almost the same doses with respect to kidney fixing sites as those of undigested IgG₁ required to induce a similar immediate proteinuria. As mentioned before, Sandberg *et al.* (1971) reported that the complement interaction site of guinea pig IgG₁ globulin was located on the $F(ab')_2$ fragments as demonstrated by the full retention of activity after digestion with pepsin. In this experiment, no rat $\beta 1c$ globulin was seen in GBM of rats given $F(ab')_2$ of IgG₁, neither was accumulation of neutrophils in glomeruli apparent, and the wide detachment of endothelial cells from GBM was the only abnormal finding. From these results, it is unlikely that the alternate pathway of complement fixation has a great significance in glomerular injuries, at least, of the initial phase of rat Masugi nephritis. The differences in the nephritogenicity between these two kinds of $F(ab')_2$ fragments, those from guinea pig IgG₁ and rabbit IgG, can not be explained at present. One possible explanation for this difference, however, may be that the fixation of pepsin digested, divalent fragments of nephrotoxic guinea pig IgG₁ antibodies to the glomerular basement membrane will activate some unknown mediators, resulting in the detachment of endothelial cells and enhancement of permeability for protein without detectable participation of the complement-leucocyte system. Such concept may be supported by facts that immune complexes could activate the kinin system (Movat and Diloranzo, 1968; Movat *et al.*, 1968), or histamine and serotonin (Kniker and Cochrane, 1968; Cochrane, 1971), followed by enhancement of vascular permeability, and that perfusion of the fenestrated capillaries of the intestinal mucosa of rats with histamine resulted in detachment of the endothelium from the basement membrane (Clementi and Palade, 1969).

The detachment of endothelial cells from GBM was observed in rats injected with nephrotoxic guinea pig IgG₁ (Kobayashi *et al.*, 1973) or rabbit IgG (Shige-

matsu, 1970). It has been considered that the detachment of endothelial cells from GBM in nephrotoxic nephritis may be due to infiltration of neutrophils in glomeruli (Cochrane, 1967) or deposition of fibrin beneath the endothelial cells (Vassalli and McCluskey, 1964). In this study, however, it is indicated that the detachment of endothelial cells from GBM is induced only by the fixation of divalent fragments of nephrotoxic guinea pig IgG₁ to GBM.

Although intraluminal coagulation was striking in glomeruli of rats injected with intact nephrotoxic guinea pig IgG₁ (Kobayashi, *et al.*, 1973), no apparent fibrin formation despite of small amounts of accumulation of platelets in glomerular capillary lumens was produced by $F(ab')_2$ fragments of IgG₁. It is probable that the acceleration of blood coagulation produced by intact nephrotoxic IgG₁ was due to the presence of Fc portion. The faster amelioration of glomerular lesions observed in rats given $F(ab')_2$ of IgG₁ may, in part, be accounted for by the lack of intraluminal coagulation. On the basis of these results, it is suggested that the glomerular injuries induced by intact nephrotoxic IgG₁ or digested fragments of IgG₁ are caused by unknown non-complement mediated factors which may be intrinsically possessed by guinea pig IgG₁ antibody molecule.

It was reported that papain treated nephrotoxic rabbit IgG was fixed to the kidney with a shorter half-disappearance time than that exhibited by the untreated nephrotoxic rabbit IgG (Stelos *et al.*, 1961). The faster disappearance of $F(ab')_2$ fragments from the kidney was confirmed in the present experiment. As demonstrated by fluorescent antibody methods, $F(ab')_2$ fragments of guinea pig IgG₁ which are probably non-nephrotoxic antibodies, were filtrated from glomeruli and reabsorbed by proximal tubular epithelial cells just readily after the injection. Furthermore, $F(ab')_2$ fragments of nephrotoxic IgG₁ that had been fixed to the glomerular basement membrane disappeared almost completely within two to three weeks after the injection. These findings may also explain the transiency of the glomerular lesions lacking the secondary phase of the disease.

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