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Enhancement of *in situ* immune complex formation in isolated perfused kidneys in Heymann's nephritis

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SUMMARY

A preparation of the putative antibody of HN (Heymann's nephritis) lacking preformed immune complexes, obtained from the acid eluates of kidneys with HN, purified through ion exchange and gel-filtration chromatography, characterized by immunoelectrophoresis, sucrose density-gradient ultracentrifugation, ^{125}I-C1q assay for immune complexes, and specific for the brush border of rat proximal renal tubule was injected intravenously, intra-arterially directly into the left renal artery of a kidney with intact normal circulation, and into the isolated buffer-perfused kidney, respectively, in three groups of rats. Controls were injected with non-antibody IgG, and antibody absorbed with the specific antigen. Localization of antibody was studied by direct immunofluorescence. Prompt localization of antibody and formation of *in situ* immune complexes occurred in the isolated perfused kidney. No localization of antibody was seen following the intrarenal arterial injection in the unperfused kidney with intact circulation, and a delay of 5 days was noted for the localization to develop upon intravenous injection. These results show that, in the homologous antibody-induced HN, the preparation of isolated perfused kidney considerably enhances the formation of *in situ* immune complexes in glomeruli as compared to the unperfused kidney with intact circulation in which the antibody is injected either directly into the renal artery or systemically through a peripheral vein.

INTRODUCTION

Because of the striking histological and immunohistological similarities between the Heymann's nephritis (HN) of rats and the idiopathic membranous glomerulonephropathy (MGN) in humans the pathogenesis of HN is of considerable interest (Heymann *et al.*, 1959; Glassock *et al.*, 1968; Alousi, Post & Heymann, 1969). The roles of the circulating immune complexes and of the *in situ* immune complexes in its pathogenesis remain controversial (Makker, Moorthy & Kirson, 1979; Neale & Wilson, 1979; Abrass, Border & Glassock, 1980; Fleuren, Ground & Hoedemaeker, 1980). Recently, with immunofluorescence technique, we have shown that when the putative antibody of homologous origin (obtained from the acid eluates of kidneys with HN) is infused into a bloodless, isolated perfused kidney, the formation of *in situ* immune complexes can be demonstrated to occur in the glomerulus (Makker & Moorthy, 1981). Similar observations using peroxidase-labelled antibody and electron microscopy have been also recently reported by Fleuren *et al.* (1980).

The present investigation was conducted to see whether the *in situ* immune complex formation could also be demonstrated in the non-isolated perfused kidney. Three groups of rats were injected with the putative antibody. In one group it was given intravenously in intact animals, in another

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group it was given directly into the left renal artery of a kidney with intact normal circulation, and in the third group it was injected into the isolated, buffer-perfused kidney.

MATERIALS AND METHODS

Preparation of rat brush-border antibody (BBAb). Rat renal cortical fraction (Fx1A) was prepared from Sprague–Dawley (SD) or Lewis rat (Charles River Breeding Laboratories Inc., Wilmington, Massachusetts) renal cortices as previously described by Edgington, Glassock & Dixon (1968). Earlier, HN was induced in SD and Lewis rats by immunizing the animals with rat kidney cortex and Freund's complete adjuvant (FCA) as originally described by Heymann *et al.* (1959). In this method rats are immunized with rat kidney cortex emulsified in FCA and multiple (6 to 10) immunizations are given intraperitoneally at 2-week intervals, and once the rats develop significant proteinuria (40 mg/17 hr of urine) the immunizations are discontinued. Over 90% of SD rats and 100% of Lewis rats develop heavy proteinuria (100–300 mg/17 hr) and have typical deposits of IgG and C3 in their kidneys. SD rats were always immunized with SD renal cortex and Lewis with Lewis renal cortex. Lately, we have induced HN in Lewis rats (Charles River Breeding Laboratories) by using SD rat Fx1A (10–20 mg) and FCA as described by Glassock *et al.* (1968). A large number of kidneys from animals with severe HN (heavy proteinuria and deposits of IgG and C3) had been collected in Dr Heymann's laboratory over many years and had been stored at −50 to −70°C. Batches of kidneys were slowly thawed at 4°C and BBAb eluted from the renal cortices by the acid elution technique using 0·03 M citric acid, sodium citrate buffer, pH 3·2, as previously described (Grupe & Kaplan, 1969; Makker & Heymann, 1973). The BBAb in the acid eluate was purified by isolating the IgG fraction by ion-exchange chromatography on DEAE–cellulose (DE-52, Whatman Inc., Clifton, New Jersey) with 0·015 M phosphate buffer, pH 7·5. To ensure that the above preparation of BBAb did not contain immune complexes, it was further purified by collecting the 7S peak by gel-filtration chromatography on Sephadex G-200 (Pharmacia Fine Chemicals), using 0·015 M phosphate buffer, pH 7·5. The 7S nature of this material was further ascertained by sucrose density-gradient ultracentrifugation using a 15–45% sucrose gradient and normal rat IgG as a marker for molecular weight as previously described (William & Chase, 1968). The BBAb so prepared was then subjected to immunoelectrophoresis along with normal SD rat serum as a control. Precipitation lines were then developed with rabbit anti-rat whole serum (Cappel Laboratories, Downington, Pennsylvania) in the trough. This material was found to be rat IgG and did not contain any other serum proteins. The preparation was also tested for preformed immune complexes and aggregated IgG by the ^{125}I-C1q binding test for immune complexes as previously described (Zubler *et al.*, 1976). The antibody was then tested for its specificity for bush-border antigen (BBAg) by indirect immunofluorescence as previously described (Makker & Heymann, 1973). The protein content of individual preparations determined by Lowry's method (Lowry *et al.*, 1951) ranged from 1·9 to 9·8 mg/ml.

Normal rat IgG. Normal rat IgG was obtained from Miles Research Products, Elkhart, Indiana. It was further purified by ion-exchange chromatography on DEAE–cellulose with 0·015 M phosphate buffer, pH 7·5, and gel-filtration chromatography on Sephadex G-200 using 0·015 M phosphate buffer, pH 7·5. The specificity of normal rat IgG was proven by immunoelectrophoresis. It was also tested for preformed immune complexes and aggregated IgG by the ^{125}I-C1q binding test for immune complexes. The protein content of this preparation was determined by Lowry's method (Lowry *et al.*, 1951).

Ex-vivo *perfusion.* Male SD rats, weighing 200–300 g, were anaesthetized with ether. Through a midline incision, the aorta and vena cava were exposed by blunt dissection. After ligation of the tributaries, temporary ligatures were placed on the aorta above the level of the left renal artery, leaving the circulation to the right kidney undisturbed. Ligatures were also placed on the aorta 2 cm below the level of the left renal artery, and on the left renal vein close to the vena cava. Through a puncture hole in the descending aorta below the level of the temporary ligature, a thin polyethelene catheter (1 mm internal diameter) was introduced and advanced to the origin of the left renal artery. A small puncture hole was made in the left renal vein to drain the blood and perfusion fluid. The

kidney was perfused with oxygenated, warm (37–38°C), phosphate-buffered saline (PBS), pH 7·2, till no visible blood escaping from the punctured renal vein could be seen. The perfusion was carried out at a rate of 20 ml/min with the help of a peristaltic pump (Buchler Peristalsis Pump, Fort Lee, New Jersey) for varying lengths of time. The kidney was then perfused slowly (1–2 min) with BBAb (0·5–10 mg) or other reagents dissolved in PBS (Table 1). This was followed by perfusion with 5 ml of PBS to wash off the unbound antibodies. The kidney was then removed and processed for immunofluorescence studies. The total duration of ischaemia prior to the injection of antibody ranged from 24 to 34 min.

Intravenous injection of BBAb. Right unilateral nephrectomy was performed under ether anaesthesia in a group of eight male SD rats 6–8 weeks old and weighing 150–200 g. Three days later, 1–30 mg of BBAb (0·5 ml) was given intravenously through the tail vein under light ether anaesthesia. Sequential open renal biopsies of the remaining kidney were performed at 10 min and 1, 4, 12, 24, 48, 72, and 96 hr, and on days 5, 10 and 56. Either the same animal or multiple animals in the same group were used for sequential biopsies. Biopsy tissue was studied by direct immunofluorescence for localization of rat IgG and C3 in the kidney. The purpose of the unilateral nephrectomy was to increase the chances of antibody deposition.

Intrarenal arterial injection of BBAb into the renal artery. Male SD rats weighing 200–300 g were anaesthetized with ether. Through a midline incision, the aorta and vena cava were exposed by blunt dissection. Ligatures were placed on the descending aorta 2–3 cm below the origin of left renal artery. Through a small puncture hole in descending aorta below the level of the ligature, a thin (0·5 mm internal diameter) polyethelene catheter was advanced to the origin of left renal artery and then negotiated into the lumen of the renal artery and advanced to the hilum of the kidney. The catheter in this location completely occludes the blood supply to the kidney and can be used for the infusion of buffer or BBAb. The accuracy of the direct renal arterial perfusion technique was confirmed by the injection of Evan's blue. No back flow of the perfusate (buffer, Evan's blue) is seen if the catheter is in the above-mentioned position and is of the size (diameter) tightly fitting the lumen of the renal artery. With practice, an injection of the antibody can be given directly into the renal artery at its entry into the kidney in a few seconds. Once the catheter is in the right position, the kidney is first quickly (5–10 sec) perfused with 10 ml of PBS and then with the antibody. Following perfusion with the antibody the catheter is quickly withdrawn into the descending aorta restoring normal circulation to the left kidney as evident by an immediate red flush in the kidney. The total ischaemia time ranges from 1 to 2 min. Renal biopsies were then obtained at 10 and 60 min and studied for localization of rat IgG by direct immunofluorescence.

Immunofluorescence technique. Renal tissue fragments were snap-frozen in isopentane, precooled in liquid nitrogen, and cut 2–4 μm thick in a cryostat. The sections were air-dried for 1 hr, and washed twice for 10 min each with PBS, pH 7·2. The sections were then reacted with fluorescein isothiocyanate (FITC) labelled goat anti-rat IgG (Travenol Laboratories, Costa Mesa, California) for 1 hr at room temperature in a moist chamber. Following this reaction, the sections were then washed again twice for 10 min each in PBS, and mounted in 1:1 solution of PBS and glycerol. Staining for C3 was performed in a similar manner using FITC-labelled rabbit anti-rat C (Cappel Laboratories, Downington, Pennsylvania). All sections were examined under the fluorescence microscope with epi-illumination (Leitz, Model Dialux). The specificity of FITC-labelled goat anti-rat IgG and rabbit anti-rat C3 was proven by immunoelectrophoresis against normal rat serum.

Absorption of rat BBAb with rat Fx1A. Brush-border antibody was absorbed with rat Fx1A as described previously (Makker, 1977). The absorbed antibody was tested for lack of reactivity against BBAg by indirect immunofluorescence on normal SD rat kidney sections.

RESULTS

Brush-border antibody

The eluted BBAb following purification through ion-exchange and gel-filtration chromatography, and sucrose density-gradient ultracentrifugation was found to be of molecular weight identical to

that of monomeric IgG and did not contain heavier particles. The results of the ^{125}I-C1q binding test for immune complexes expressed in μg/ml of aggregated human gammaglobulin equivalents were as follows: normal SD rat serum 68–84, normal rat IgG fractions 70–82, and BBAb fractions 72–85. These results show that both the normal rat IgG fraction and the BBAb fraction were similar to normal rat serum as far as the presence of pre-formed immune complexes and the aggregated IgG are concerned. The results of gel-filtration, sucrose density-gradient ultracentrifugation, and of the ^{125}I-C1q binding test for immune complexes clearly show that the BBAb fraction was free of aggregated IgG or preformed immune complexes.

The BBAb was found to be IgG by immunoelectrophoresis and reacted specifically with the brush-border region of the proximal renal tubules by indirect immunofluorescence (Fig. 1). No appreciable reaction was noted with the glomerular components. The specificity of the BBAb was further confirmed by its susceptibility to be completely absorbed by rat Fx1A. Usually, 8–10 mg of Fx1A was required to absorb 1 mg of BBAb.

Ex-vivo *perfusion*

Four groups of animals were perfused with normal rat IgG, rat BBAb IgG, rat BBAb absorbed with rat Fx1A, *or* PBS alone, respectively. These data are summarized in Table 1.

(1) Perfusion with BBAb. Direct immunofluorescence of the kidneys, perfused with BBAb in the absence of blood and thus of any possible circulating BBAg, showed fine to coarse granular fluorescence in the glomeruli (Fig. 2). The glomerular involvement was focal and segmental, and the fluorescence was primarily seen along the capillary loops, although it was also present in mesangium. In some areas, staining of the brush border of the proximal tubules was also seen. In addition to the glomeruli, prominent fine to coarse granular fluorescence was also seen in the walls of blood vessels. The involved vessels were: afferent arterioles, capillaries in interstitium, and small- and medium-sized vessels (Fig. 3). No staining was seen in the interstitium. The staining in glomeruli and vessels could be seen with as little as 0·5 mg of BBAb.

(2) Control perfusions. Kidneys perfused with PBS, normal rat IgG, or with rat BBAb previously absorbed with rat Fx1A did not show any staining for rat IgG in the glomeruli, vessels, tubules or any other structures.

Immunofluorescence studies of the unperfused normal right kidney also did not reveal any staining for rat IgG.

Intravenous injection of BBAb

These results are summarized in Table 2. No deposits of rat IgG were seen in the kidney till 96 hr after the injection of the antibody. Minimal deposits were first clearly seen only after 5 days of the

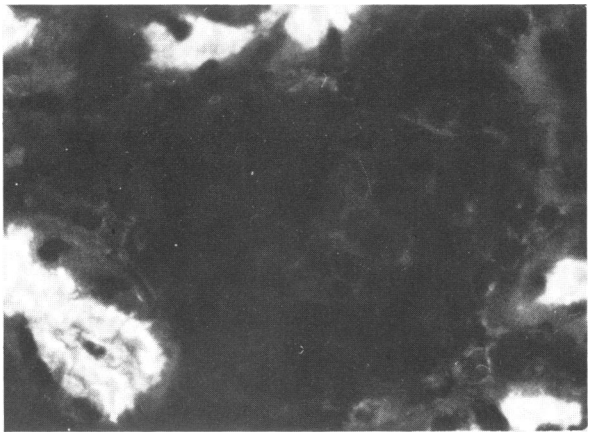

Fig. 1. Reaction of BBAb with proximal renal tubules studied by indirect immunofluorescence on normal Sprague–Dawley (SD) rat kidney section. Bright fluorescence is seen in the brush-border region of the renal tubules. (Original × 500.)

Table 1. Brush-border antibody localization in isolated perfused kidney

Rat no.	Perfused substance	Dose (mg)	Ischaemia (min)	BBAb localization			
				Glomerulus			Tubular BB
				Loop	Mesangium	BV	
1	Normal rat IgG	5	31	−	−	−	−
2	Normal rat IgG	10	30	−	−	−	−
3	Normal rat IgG	10	34	−	−	−	−
4	Normal rat IgG	20	32	−	−	−	−
5	Normal rat IgG	20	28	−	−	−	−
6	Rat BBAb	0·5	30	+	+	+	+
7	Rat BBAb	1	32	+	+	+	±
8	Rat BBAb	2	26	+	+	+	±
9	Rat BBAb	5	28	+	+	+	+
10	Rat BBAb	10	24	+	+	+	+
11	Fx1A absorbed BBAb	1/8*	28	−	−	−	−
12	Fx1A absorbed BBAb	2/10*	26	−	−	−	−
13	PBS	—	30	−	−	−	−
14	PBS	—	26	−	−	−	−

BV = blood vessels, Tubular BB = brush-border are of proximal renal tubules.
* BBAb mg 1/Fx1A mg 8, BBAb mg 2/Fx1A mg 20.
− = No fluorescence, + = positive fluorescence, ± = trace to absent.

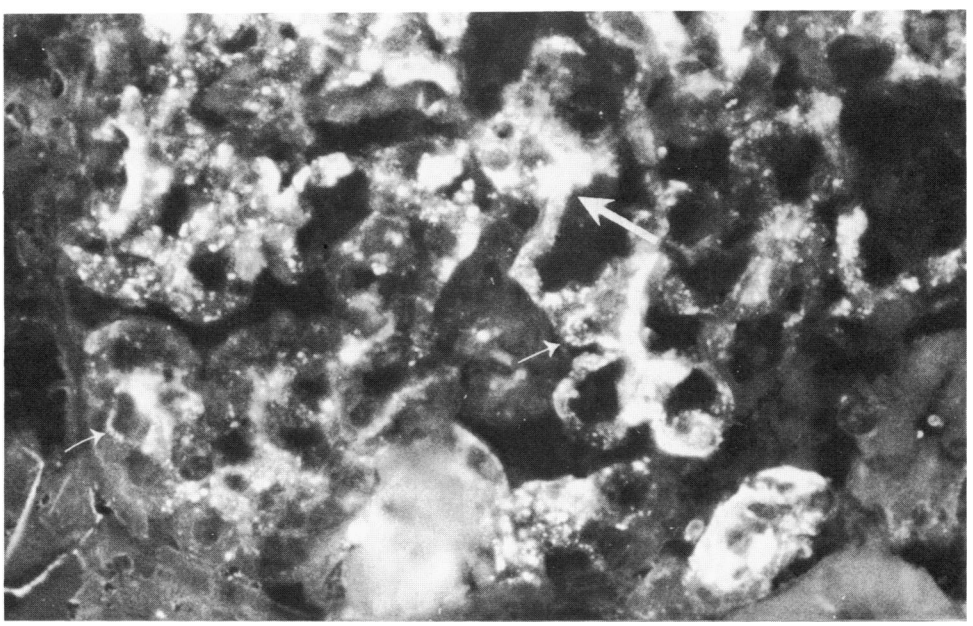

Fig. 2. Kidney section from isolated perfused kidney perfused with BBAb and studied by direct immunofluorescence for localization of rat IgG. Granular staining along the capillary loops of a glomerulus in many loops is prominent (*thin arrows*) and in some areas mesangial staining (*thick arrows*) is also present. (Original × 720.)

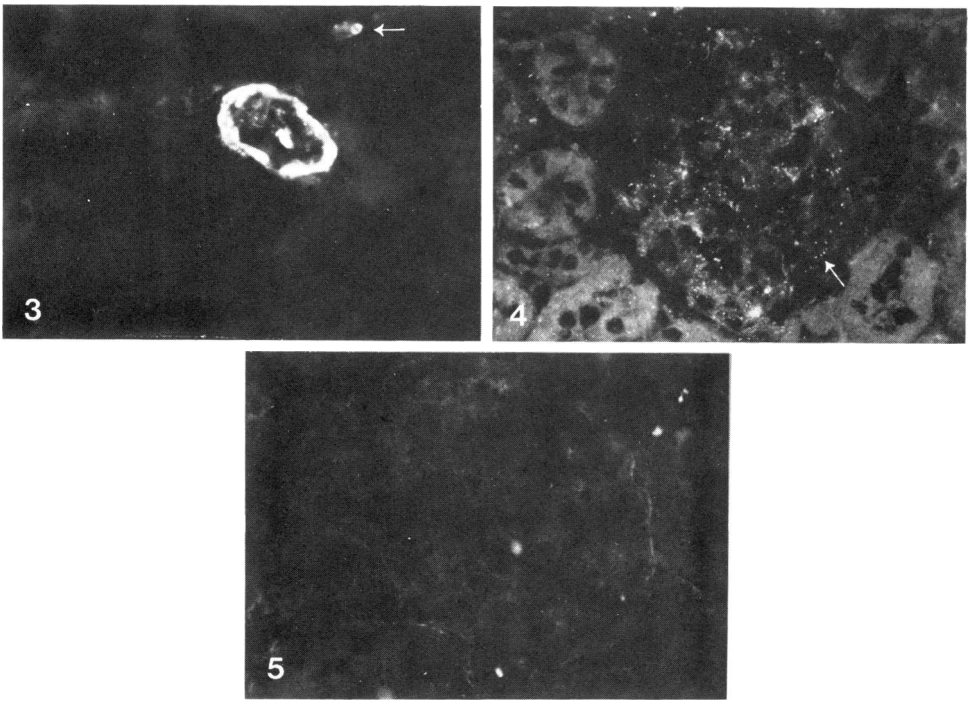

Fig. 3. Kidney section from an isolated perfused kidney perfused with BBAb shows localization of the antibody in the wall of a vessel. Granular staining in a peritubular capillary (*arrow*) is also present. (Original × 720.)

Fig. 4. Kidney section from an animal injected intravenously with BBAb and studied on the 10th day by direct immunofluorescence for the localization of rat IgG. Discrete fine granular staining is seen along the capillary loops (*arrow*). (Original × 375.)

Fig. 5. Kidney section from an animal intrarenally perfused with BBAb arterially shows only minimal background fluorescence. (Original × 500.)

Table 2. Brush-border antibody localization following intravenous injection

Rat no.	Dose (mg)	Time of biopsy	BBAb localization* in glomeruli
1	1	10 min, 24 hr	−
2	5	60 min, 24 hr	−
3	30	10 min, 4 hr	−
4	30	1, 12, 24 hr	−
5	30	Days 1, 3, 5	+day 5
6	30	Days 2, 4, 10	+day 10 ±day 4
7	30	Days 5, 10	+days 5, 10
8	30	Days 5, 10	+days 5, 10

* BBAb localized along the glomerular capillary loops only.

− = No fluorescence, + = positive.

Table 3. Brush-border antibody localization following renal arterial infection

Rat no.	Dose (mg)	BBAb localization in kidney
1	0·5	–
2	1	–
3	10	–
4	30	–
5	30	–

– = No fluorescence.

injection and seemed to increase on the 10th day (Fig. 4). These deposits were granular, but sparse and discrete and were not accompanied by any staining for rat C3. No deposits for rat IgG were present in glomeruli on the 56th day.

Intrarenal arterial injection of BBAb

These results are summarized in Table 3. No staining for rat IgG was seen in any of the glomeruli in any animal injected with 0·5–30 mg of BBAb (Fig. 5) in the renal biopsies obtained at 10 and 60 min. The ischaemia time to the kidney in each instance was less than 3 min.

DISCUSSION

The characteristics of the antibody preparation used in our experiments were its homologous origin, the residence of its activity in the IgG fraction, its specificity for the brush border of the proximal renal tubule, and its lack of contamination with particles heavier in molecular weight than IgG, aggregated IgG and preformed immune complexes.

Since the concentration of BBAg in blood is extremely small (Glassock *et al.*, 1968), and since the kidney in the isolated perfused case is well perfused (500–600 ml of buffer over 24–32 min), it is extremely unlikely that there is a sufficient amount of BBAg of circulatory origin left in the perfused kidney to form immune complexes with the infused antibody. This most probably rules out the possibility that the localization of antibody in the glomeruli in the isolated perfused kidney represents trapping of the preformed immune complexes formed with the antigen from circulation. The glomerular localization of BBAb is also not due to a non-specific reaction or a mere entrapment of the IgG molecule in the glomerular structures peculiar to the isolated perfused kidney, because no such localization was observed in control kidneys when non-BBAb IgG molecules of similar characteristics were infused under similar conditions. Furthermore, the infused antibody (BBAb) did not lose its specificity for BBAg, in addition to the staining in glomeruli, staining of the tubular brush border was also clearly visible. These findings suggest that the staining with BBAb is most likely a specific antigen–antibody reaction and thus is an example of *in situ* immune complex formation.

This phenomenon of *in situ* immune complex formation along the capillary loops, using heterologous anti-Fx1A antibody and a similar perfusion set-up and immunofluorescence methodology, has been earlier demonstrated by Van Damme *et al.* (1978) and Couser *et al.* (1978). Also, in an improved version of the perfusion set-up, Couser *et al.* (1978) perfused isolated rat kidneys at controlled perfusion pressure, pH, temperature and flow rates with recirculating oxygenated perfusate containing bovine serum albumin and heterologous anti-Fx1A antibody in Krebbs–Henseleit bicarbonate buffer. No significant differences in the *in situ* phenomenon were observed between the two perfusion methods by Couser *et al.* demonstrating the fact that the simple perfusion set-up used by us and Van Damme *et al.* is adequate to demonstrate the phenomenon of *in situ* immune complex formation.

The localization of the infused BBAb in the walls of the vessels and mesangium of some glomeruli is of interest. It is unlikely that it is due to the injection and entrapment of preformed immune complexes or aggregated IgG at these sites because (1) the antibody fraction as characterized by gel-filtration and sucrose density-gradient ultracentrifugation did not contain particles that were larger than IgG in size, and (2) the ^{125}I-C1q binding test for immune complexes did not show abnormal amounts of preformed immune complexes or aggregated rat IgG in the BBAb fraction. Moreover, no staining in glomeruli or blood vessels was seen in control kidneys perfused with normal rat IgG fraction similarly characterized as the BBAb fraction and that had similar values with the ^{125}I-C1q binding test or immune complexes as the antibody fraction. Couser *et al.* (1978), in their study with heterologous anti-Fx1A antibody, also noted minimal mesangial staining in rare glomeruli but did not mention whether any staining was present in the vessels. Van Damme *et al.* (1978), also using heterologous anti-Fx1A antibody, noted reaction along the capillary walls of glomeruli and brush border of some proximal tubules and the walls of capillaries in the interstitium. These investigators did not mention whether they observed any staining in larger vessels and mesangium. Fleuren, Ground & Hoedemaeker (1980), in their recent study with peroxidase-labelled homologous antibody and immunoelectron microscopy, noted reaction along the entire capillary wall (endothelium, basement membrane and epithelial foot processes). No mention was made of the reaction in other blood vessels. In our study the staining in the walls of the vessels of varying size was quite prominent suggesting that the antigen may also be present in the walls of the other intrarenal vessels in addition to the glomerular capillaries.

In the intravenously injected animals, we found that the antibody localization in the glomeruli first became apparent on the 5th day and increased on the 10th day. Similar results were obtained by Sugisaki *et al.* (1973) in their study where the antibody was injected intraperitoneally. These investigators gave five to seven injections on alternate days and found small amounts of localization in the glomeruli on the 9th day. Obviously, in the intravenously injected animals, the injected antibody is immediately diluted within the circulation and probably then slowly localizes in the kidney, gradually increasing in amount over time after several passes through the kidney. The minimal earlier localization is apparently not detected because of the relative insensitivity of the direct immunofluorescence technique. As more and more localization occurs and the amount of antibody increases at a given site, it then falls within the range of the sensitivity of the direct immunofluorescence technique, and, therefore, can be detected to increase between days 5 and 10. Whether this localization in the glomerulus represents complexes formed *in situ* or complexes earlier formed in circulation and subsequently trapped in glomerulus cannot be answered from this data. Presumably, once the antibody is depleted from the circulation, the antibody localized in the kidney is gradually resolved and is no longer detectable at 8 weeks. Early (10 min) glomerular localization of heterologous (rabbit and sheep) anti-rat Fx1A antibody following intravenous administration has been demonstrated by Van Damme *et al.* (1978) and Couser *et al.* (1978). However, it should be pointed out that the above investigators used heterologous antibodies and the antibodies were prepared against fraction Fx1A, which is a crude renal cortical fraction, and most likely contains *a number of* antigens. These differences may account for the variance in the results between experiments with heterologous *vs* homologous antibodies.

Failure of the antibody to localize in the kidney following a direct intrarenal arterial injection, as opposed to its prompt localization in the isolated perfused kidney, is surprising. It is certainly not due to the insufficient amount of antibody injected in the case of intrarenal arterial experiment, because injection of as little as 0·5 mg of antibody in the isolated kidney produced a definite localization, wheras up to 30 mg of antibody in the intrarenal arterial experiments did not produce any localization. It is also not due to a slower flow in the isolated perfused kidney because the perfusion flow in the above kidney was five to six times greater than the *in vivo* renal plasma flow in the normal kidney. Since all other variables (age, sex and strain of animals, quality of antibody, buffers and reagents, technical aspects, etc.) were also controlled, the explanation for the variance in results between the two experiments must reside in the nature of the two preparations—isolated perfused versus unperfused kidney. Clearly, the antigen is more readily accessible to the antibody in the isolated perfused kidney; however, which factors in the latter improve this accessibility is not apparent. A major difference between the two preparations is the duration of ischaemia which may

be one such factor. There may, however, be additional differences between the two preparations which are not readily apparent and may also contribute towards the variance.

Our results do not completely resolve the present controversy regarding the pathogenesis of HN. The results in the isolated perfused kidney clearly show the *in situ* formation of immune complexes along the capillary loops of the glomerulus upon injection of the putative antibody without the exogenous antigen. In addition, these results suggest the presence of the same or a cross-reacting antigen in the wall of other intrarenal blood vessels. The results in the intravenously injected animals can be interpreted to favour either of the proposed mechanisms of pathogenesis (*in situ vs* circulatory immune complexes), although the delay (5 days) in the localization may be more in favour of the latter mechanism. The results of intrarenal arterial infusion do not demonstrate the formation of *in situ* complexes and seem to be more in favour of the circulatory–complex mechanism; however, whether in the physiologically perfect *in vivo* state the complexes never develop *in situ* cannot be ruled out. It seems more likely that both the *in situ* and the circulatory mechanisms are involved in the pathogenesis of HN as has also been recently suggested by Abrass, Border & Glassock (1980) who have demonstrated immune complexes containing the offending antigen and antibody in the circulation of rats with HN.

Our studies, however, convincingly demonstrate enhanced binding of the antibody and thus of the formation of *in situ* immune complexes in the isolated perfused kidney as compared to an unperfused kidney with intact circulation. The mechanism for this enhancement has not been elucidated and will require further studies.

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