QUANTITATIVE STUDIES OF *IN SITU* IMMUNE COMPLEX GLOMERULONEPHRITIS IN THE RAT INDUCED BY PLANTED, CATIONIZED ANTIGEN*

By T. OITE, S. R. BATSFORD, M. J. MIHATSCH, H. TAKAMIYA, AND A. VOGT

From the Institute of Immunology, Zentrum für Hygiene der Universität Freiburg, 7800 Freiburg im Breisgau, Federal Republic of Germany, and the Institut für Pathologie der Universität Basel, 4056 Basel, Switzerland

Recent experimental studies have shown that the local binding of circulating antibody with antigens existing or planted within the glomerulus can result in damage to this structure (1–6). In particular, chemically cationized proteins are able to bind to the glomerular basement membrane (GBM), act as a planted antigen, and induce *in situ* immune complex formation accompanied by marked proteinuria (4–6). Negatively charged components in the GBM (7–10) and on the surfaces of endothelial and epithelial cells (11–14) act as a barrier to the passage of anionic, but not cationic macromolecules (15, 16). These fixed anionic sites also function as receptors for cationic substances, and this is the basis of the system developed.

We describe the first detailed quantitative studies in a model of *in situ* immune complex nephritis that uses a cationic antigen, cationized human immunoglobulin G (IgG). The correlation between the degree of proteinuria and the amount of kidney fixing antigen and antibody was investigated. In addition, the distribution of cationized antigen within the organism and the disappearance kinetics of antigen and antibody from the kidney were analyzed. The results documented here provide insight into events occurring at a molecular level during immune complex formation involving cationic antigens, which is an essential step before attempting to extrapolate the concept of charge-based complex localization to man.

Materials and Methods

Rats. All experiments were performed on male Wistar rats of body weight varying from 120 to 150 g (Zentraltierzüchterei, Hannover, Federal Republic of Germany).

Preparation of Cationized Human IgG and Determination of the Isoelectric Point (pI). Cationization of human IgG was performed as described previously (5), based on the method of Danon et al. (17) using 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Chemical Co., St. Louis, Mo.) as activator and *N,N*-dimethyl-1,3-propanediamine (DMPA) (Eastman Kodak Co., Rochester, N. Y.) as a nucleophile to replace carboxyl groups. 2 ml DMPA was added to 40 ml distilled water, and the pH was adjusted to 6.5 with 1 N HCl. 1.6 g human IgG (10 ml Beriglobin, Behringwerke, F. R. G.) and 3 g EDC were added under mild stirring. The pH was maintained at 6.5 for 6–8 h in a TTT 60 Titrator (Radiometer,

* This work was supported by the Dentsche Forschungs gemeinshaft Vo 91/16. Dr. Oite was the recipient of an Alexander von Humboldt foundation fellowship.

Abbreviations used in this paper: BSA, bovine serum albumin; Con A, concanavalin A; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycan; GBM, glomerular basement membrane; PBS, phosphate-buffered saline; pI, isoelectric point.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/02/0460/15 $1.00
Volume 155 February 1982 460–474

Copenhagen, Denmark) using 0.2 N HCl. The reaction mixture was left overnight at room temperature, dialyzed against six changes of phosphate-buffered saline (PBS) in the cold, sterile filtered through a millipore filter (0.45 μm), and stored at 4°C until required. The degree of cationization was estimated from isoelectric focusing in a slab gel system (Desaga, Heidelberg, F. R. G.). The reaction conditions given produced cationized human IgG with a pI exceeding 9.5, and this was used throughout the study. To check for the presence of aggregated material, the cationized human IgG was run on 7.5% polyacrylamide gel using a basic gel system (18). Staining with amidoschwarz indicated that the cationized preparation consisted of 90% monomer, ~5% dimer, and 1–2% larger polymers. Analysis by gel column filtration was less satisfactory, because the material apparently interacts with the gel matrix, as reported by other workers (15).

Antisera. Antisera to human IgG were raised in rabbits by repeated injections of antigen in completed Freund's adjuvant. All anti-human IgG antibody used was from a single pool, with an antigen-binding capacity of 1280 μg/ml, measured by the P-80 method (19). The IgG fraction of this pool was isolated by repeated ammonium sulphate precipitation.

Radiolabeling. Cationized human IgG and the IgG fraction of rabbit anti-human IgG were labeled with ^{125}I and ^{131}I, respectively, by the chloramine-T method (20). Labeled preparations in which >95% of the isotope was protein bound were used. The specific activity was 1×10^7 to 3×10^7 cpm/mg of ^{125}I-labeled cationized human IgG and 3×10^6 to 4×10^6 cpm/mg of ^{131}I-labeled anti-human IgG. Before use, the preparation was diluted with the appropriate amount of unlabeled material that had been treated with chloramine-T. Total amounts of ^{125}I-labeled cationized human IgG injected were equivalent to 6×10^4 to 8×10^4 cpm/animal, and in the case of ^{131}I-labeled anti-human IgG an equivalent of 1×10^5 to 1.5×10^5 cpm/animal was injected. Radioactivity was determined in a well-type gamma counter Berthold, 7547 Wildbad, F. R. G. (LB MAG 3/2; Berthold, Wildbad, Federal Republic of Germany).

Unilateral Renal Perfusion. A number 23 needle (Record) was used to canulate the left renal artery, and the left kidney was perfused with 0.5 ml PBS, followed by the test material in a volume of 0.2 ml, both at a rate of 0.5 ml/min, after which the tubing was flushed with an additional 0.1 ml PBS. The period of ischemia ranged from 4 to 7 min.

Estimation of Proteinuria. 24-h urinary specimens were collected in metabolic cages, centrifuged at 1000 *g* for 10 min, and the protein content was measured with the biuret method (21) using human serum albumin as standard.

Histological Examination. For light microscopy, renal tissue was fixed in buffered formalin, embedded in paraffin, and 4-μm sections were stained with hematoxilin-eosin, periodic acid-Schiff, or periodic acid silver methamine. For immunofluorescence, material was snap-frozen in n-hexane cooled to −70°C. Frozen sections were stained with monospecific fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Behringwerke), anti-rabbit IgG, anti-rat IgG, and anti-rat C3 (Nordic Immunochemicals, Tilberg, Holland). Tissue fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was processed for electron microscopy as described by Zollinger and Mihatsch (22).

Experimental Design. Five groups of experiments were performed according to the following schedules; three to five rats were used per experiment at each time point studied.

1. DISTRIBUTION OF CATIONIZED HUMAN IgG AFTER INTRAVENOUS INJECTION. Rats were given 1 mg of ^{125}I-labeled cationized human IgG in 1 ml PBS and killed 1 h later, and the activity in the left and right kidney and in samples of blood, spleen, liver, lung, and heart was determined.

2. DISTRIBUTION OF CATIONIZED HUMAN IgG AFTER INTRARENAL INJECTION. The left kidneys of rats were perfused with 5–200 μg of ^{125}I-labeled cationized human IgG. Samples were measured as in experiment 1.

3. NEPHRITOGENIC POTENTIAL OF CATIONIZED HUMAN IgG PLANTED IN THE GLOMERULUS AFTER INTRAVENOUS ADMINISTRATION OF ANTI-HUMAN IgG ANTISERUM. 5–100 μg of cationized human IgG was perfused directly into the left kidney via the renal artery. 1 h later, the rats were injected with 0.5 ml rabbit anti-human IgG antiserum intravenously. As controls, the following two experiments were done: (*a*) perfusion of 100 μg of normal human IgG, followed by 0.5 ml of anti-human IgG intravenously 1 h later; and (*b*) perfusion of 50 μg of cationized human IgG, followed by 0.5 ml of normal rabbit serum 1 h later. Urinary protein excretion was examined

daily for a period of up to 4 wk. Renal tissue was taken for immunofluorescent study at intervals between 1 h and 3 mo, and for light and electron microscopy between 1 d and 3 mo.

4. DEGREE OF BINDING OF ANTI-HUMAN IgG ANTIBODY TO A PREVIOUSLY PLANTED, NEPHRITOGENIC DOSE OF CATIONIZED HUMAN IgG. A group of rats were perfused with 20 μg of cationized human IgG (for selection of dose, see Results) into the left renal artery, and 2 h later 1.0–10 mg of ^{131}I-labeled anti-human IgG antibody globulin was given intravenously. 72 h later, rats were killed, and the quantity of anti-human IgG in each kidney was determined. For controls, rats were perfused via the left renal artery with 20 μg of native human IgG, and 1 h later were given 2.5 mg of ^{131}I-labeled anti-human IgG antibody globulin intravenously; second, rats were injected with 2.5 mg of ^{131}I-labeled normal rabbit IgG 1 h after perfusion of the left kidney with 20 μg of cationized human IgG. The value for renal binding of ^{131}I-anti-human IgG in the left kidney was corrected for blood contamination by deducting the counts found in the right kidney. Binding of anti-human IgG to the cationized human IgG localized in the kidneys was also checked for by immunofluorescence. For these experiments, rats were perfused with up to 100 μg of cationized human IgG into the left renal artery, followed by intravenous injection of 0.5 ml of anti-human IgG 1 h later, and were killed 1 h after injection. As a control, normal rabbit serum was substituted for the anti-human IgG antiserum.

5. DISAPPEARANCE KINETICS OF CATIONIZED HUMAN IgG FROM THE KIDNEY AFTER INTRAVENOUS INJECTION. Rats were given 1 mg ^{125}I-labeled cationized human IgG via the tail vein. The animals were killed 1, 3, 6, 12, 24, 48, and 72 h after injection, and the amount of cationized human IgG localized in the kidneys was measured.

6. DISAPPEARANCE KINETICS OF CATIONIZED HUMAN IgG PERFUSED DIRECTLY INTO LEFT KIDNEY AND EFFECT OF SUBSEQUENT ADMINISTRATION OF ANTIBODY. 43 rats were perfused with 20 μg of ^{125}I-labeled cationized human IgG each into the left kidney. Groups of three to five rats were killed 1 h or 1, 3, and 5 d later. In the remaining 28 rats, 2.5 mg of rabbit anti-human IgG antibody was injected intravenously 1 h after the antigen, and they were killed 1 h or 1, 2, 3, 5, 7, 14, and 21 d later. Renal binding of cationized human IgG was measured in all animals.

7. DISAPPEARANCE KINETICS OF ANTI-HUMAN IgG ANTIBODY FROM THE KIDNEY. *In situ* immune complex formation was induced by unilateral perfusion of 20 μg of cationized human IgG, followed by intravenous injection of 2.5 mg of ^{131}I-labeled rabbit anti-human IgG antibody globulin; these conditions produced glomerulonephritis. Rats were killed after 1 h and on days 1, 2, 3, 5, 7, 14, and 21, and renally bound antibody was measured.

Results

1. Distribution of Cationized Human IgG after Intravenous Administration. The distribution of cationized human IgG 1 h after intravenous injection is shown in Fig. 1, which is ~20 μg (2%) of the total dose injected (1 mg) localized per kidney. The major site of antigen localization was the liver (20%).

2. Distribution of Cationized Human IgG after Perfusion into the Left Kidney. Results obtained 1 h after perfusion (Table I) showed that, with doses of between 5 and 50 μg cationized human IgG, the amount bound to the left kidney increased linearly and was ~50% of the total injected. In the right kidney a maximum of 0.5% of the injected dose was present (blood contamination presumably contributed to this value [Fig. 2]). In the cases of doses of 100 and 200 μg cationized human IgG, the fraction of antigen localizing in the perfused kidney fell to 33 and 23%, respectively. In parallel, the amount of antigen present in the right kidney increased, reaching 2.2% of the total injected, equivalent to a maximum of 12.5% of the quantity found in the left kidney.

Positive fluorescence along the GBM was confined to the left kidney with a dose of up to 100 μg of cationized human IgG. The interstitium was negative. A dosage of 200 μg resulted in positive immunofluorescence in the right kidney as well. Positive interstitial staining was observed in the left kidney. Fig. 2 shows the whole body

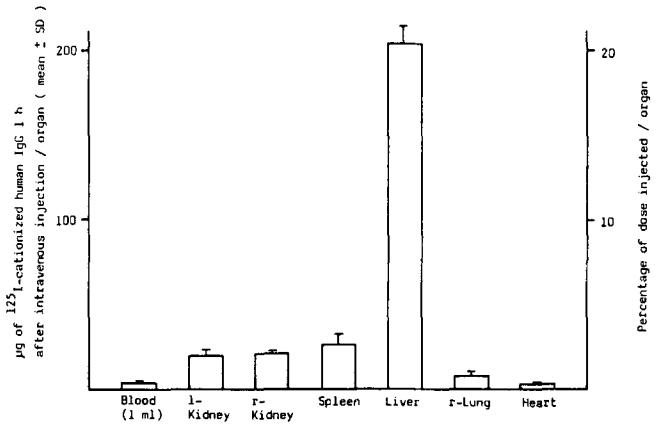

FIG. 1. Distribution of cationized human IgG given intravenously. Three rats were each injected with 1 mg ^{125}I-cationized human IgG in 1 ml PBS via the tail vein, killed ~1 h later, and the activity in the various samples was measured.

TABLE I

*Amounts of ^{125}I-cationized Human IgG Bound to Perfused Kidney**

^{125}I-cationized human IgG infused	^{125}I-cationized human IgG	
	Left kidney	Right kidney
μg	*μg/kidney*	
5	2.4 ± 0.9‡ (48)§	0.01 ± 0.003‡ (0.2)§
10	5.6 ± 0.4 (56)	0.03 ± 0.001 (0.3)
20	11.1 ± 0.8 (56)	0.07 ± 0.02 (0.4)
50	22.6 ± 4.4 (45)	0.24 ± 0.03 (0.5)
100	33.3 ± 10.6 (33)	0.64 ± 0.20 (0.6)
200	45.8 ± 19.4 (23)	4.46 ± 2.16 (2.2)

* Rats were killed 1 h after perfusion.
‡ Mean ± SD of three to five rats.
§ Percentage of dose injected, mean of three to five rats.

distribution of cationized human IgG after perfusion of 20 μg of antigen into the left kidney. The perfused kidney contained 56% of the total dose injected, compared with 2% after intravenous application (see above).

3. Nephritogenic Potential of Cationized Human IgG Planted in the GBM after Passive Administration of Anti-Human IgG Antiserum. These experiments were performed with a standard volume of antiserum (0.5 ml), which had an antigen-binding capacity of 640 μg. The degree of proteinuria induced depended on the dose of cationized human IgG previously administered. 10 μg of antigen or less produced a mild proteinuria, and rats given 20 μg or more of cationized human IgG developed a severe glomerulonephritis, with a proteinuria of at least 100 mg/24 h. The time of onset as well as the persistence of proteinuria depended on the dose of antigen perfused (Table II). No abnormal proteinuria was observed in control experiments.

Immunofluorescence revealed deposition of human IgG and rabbit IgG along the glomerular capillary wall (Fig. 3 a, b). These deposits appeared confluent on the first 2 d (Fig. 4 a) and became more granular during the course of the disease (Fig. 4 b). Moderate staining for rat C3 was seen at 1 h (the earliest time tested) and remained

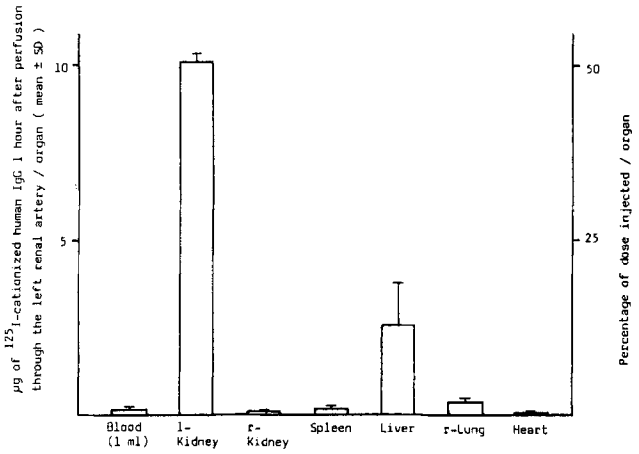

FIG. 2. Distribution of cationized human IgG perfused via the renal artery. The left kidneys of four rats were each perfused with 20 μg ^{125}I-cationized human IgG. 1 h later rats were killed, and activity in the various samples was measured.

TABLE II

Nephritogenic Potency of Renally Bound, Cationized Human IgG, after Passive Administration of Excess of Anti-Human IgG Antiserum

Unilateral renal perfusion*	Intravenous injection‡	Average mg urinary protein per 24 h§					
		Day 1	Day 2	Day 3	Day 5	Day 7	Day 14
5 μg cationized human IgG	0.5 ml anti-human IgG (rabbit)	5.0	3.9	14.2	22.4	37.8	9.4
10 μg cationized human IgG	0.5 ml anti-human IgG (rabbit)	8.2	4.5	7.2	18.7	32.9	10.9
20 μg cationized human IgG	0.5 ml anti-human IgG (rabbit)	3.8	77.4	93.0	158.5	107.2	13.1
50 μg cationized human IgG	0.5 ml anti-human IgG (rabbit)	2.3	78.3	246.7	231.8	216.8	44.5
100 μg cationized human IgG	0.5 ml anti-human IgG (rabbit)	69.0	115.0	214.0	246.6	229.0	102.0
50 μg cationized human IgG	0.5 ml normal rabbit serum	1.0	3.0	5.6	2.0	4.5	6.6
100 μg normal human IgG	0.5 ml anti-human IgG (rabbit)	3.3	4.9	3.9	8.2	6.6	ND‖

* Rats were perfused with cationized human IgG in a volume of 0.2 ml via the left renal artery.
‡ Rats were injected through the tail veins 1 h after perfusion.
§ Average of three to five rats.
‖ Not determined.

fairly constant within the first 2 wk, declining thereafter. The pattern was similar to that of the human IgG. Complement deposition clearly preceded development of proteinuria. Rat IgG was detectable in the capillary walls from approximately day 7 onwards. By this time the proteinuria was declining, and a second phase was not seen. Electronmicroscopy revealed a prominent accumulation of electron dense deposits, mainly in the subepithelial space and beneath slit pores (Fig. 5). These were already

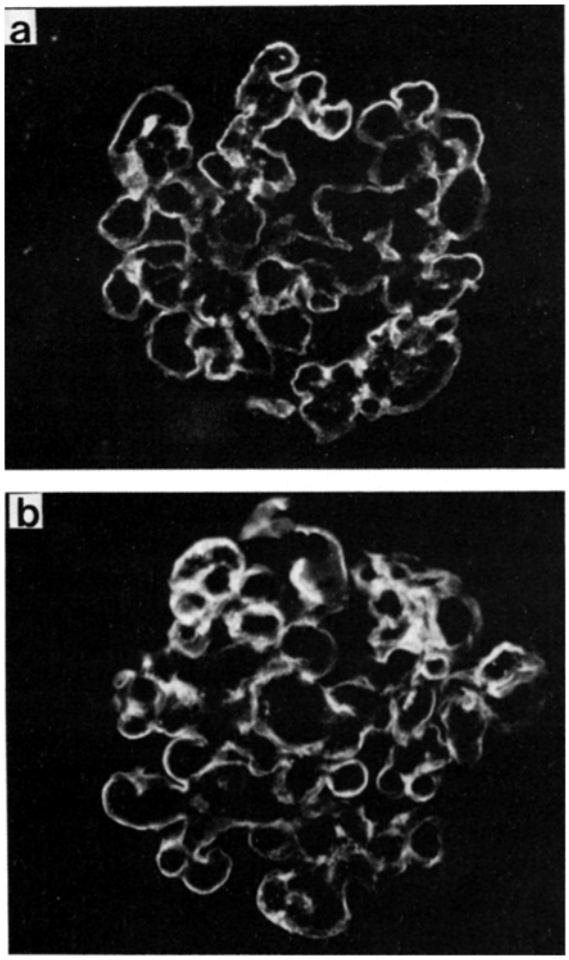

FIG. 3. Cryostat sections of rat glomeruli. (a) Stained with fluorescein isothiocyanate (FITC) anti-human IgG. 100 μg of cationized human IgG was perfused via the renal artery and rat was killed 1 h later. Glomerular localization of human IgG was seen along the capillary walls (× 200). (b) Stained with FITC anti-rabbit-IgG. Perfusion of 100 μg of cationized human IgG followed by passive injection of rabbit anti-human IgG and killing 15 min later. Rabbit IgG was found along the capillary walls in a pattern identical to human IgG (c.f. Fix. 3a) (× 200).

visible on day 1, the earliest time point investigated. Glomerular hypercellularity was not pronounced.

4. Binding of Anti-Human IgG Antibody to Planted Cationized Human IgG. 20 μg of cationized human IgG was chosen for these experiments (see Table II), and a minimum of 2.5 mg of ^{131}I-labeled anti-human IgG had to be injected intravenously to regularly induce proteinuria. In absolute values, 35.9 μg of antibody IgG was found in the left kidney on day 3, whereas only 4.0 μg was found in the right kidney, giving a corrected uptake of 31.9 μg in the left kidney (see Materials and Methods). The quantity of ^{131}I-labeled anti-human IgG antibody bound to the left kidney increased with the quantity of anti-human IgG injected (Table III). The activity in the right

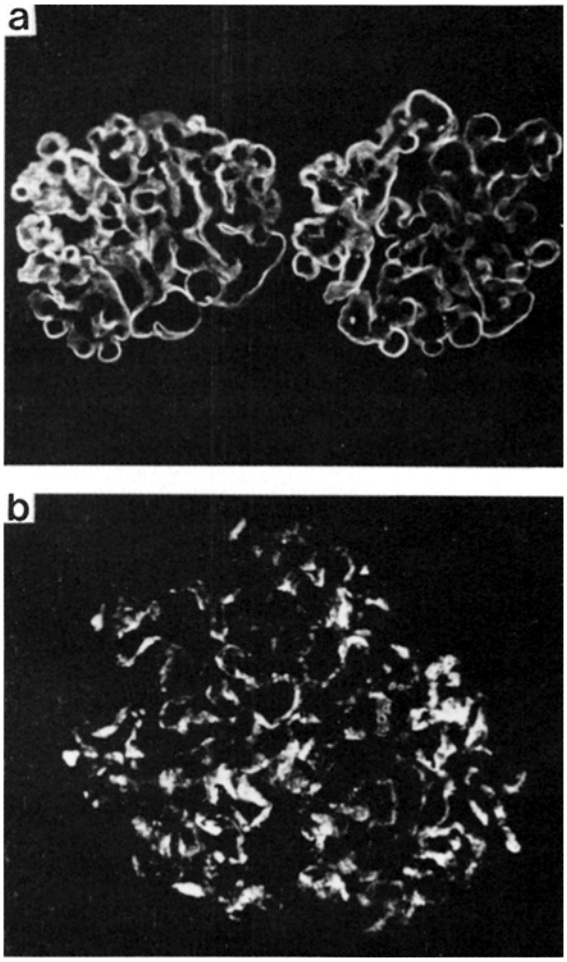

FIG. 4. Cryostat sections of rat glomeruli. (a) stained with FITC anti-human IgG. 1 d after perfusion of 100 μg cationized human IgG, followed by passive injection of rabbit anti-human IgG serum, human IgG appears to localize almost continuously along the capillary loops (× 150). (b) Stained with FITC anti-rabbit IgG. Left kidney was perfused with 100 μg of cationized human IgG, followed by passive injection of anti-human IgG antiserum. 10 d later rat was killed. Discrete granular deposition of rabbit IgG was seen along the capillary loops (× 200).

kidney corresponded to contamination with ~0.1 ml blood (Table III), an acceptable estimate confirming the suitability of the formula used.

5. Disappearance Kinetics of Intravenously Administered ^{125}I-labeled Cationized Human IgG from the Kidney. The rate of removal of intravenously injected ^{125}I-labeled cationized human IgG from the kidneys is shown in Table IV. 21.1 and 20.6 μg of cationized human IgG were bound in the left and right kidneys, respectively, 1 h after injection. There followed a relatively rapid decrease up to 6 h, with a subsequently slower rate of disappearance.

6. Disappearance Kinetics of Cationized Human IgG Perfused Directly into Left Kidney and Effect of Subsequent Administration of Antibody. Fig. 6 shows the disappearance kinetics of cationized human IgG from the perfused kidney with and without passive injection

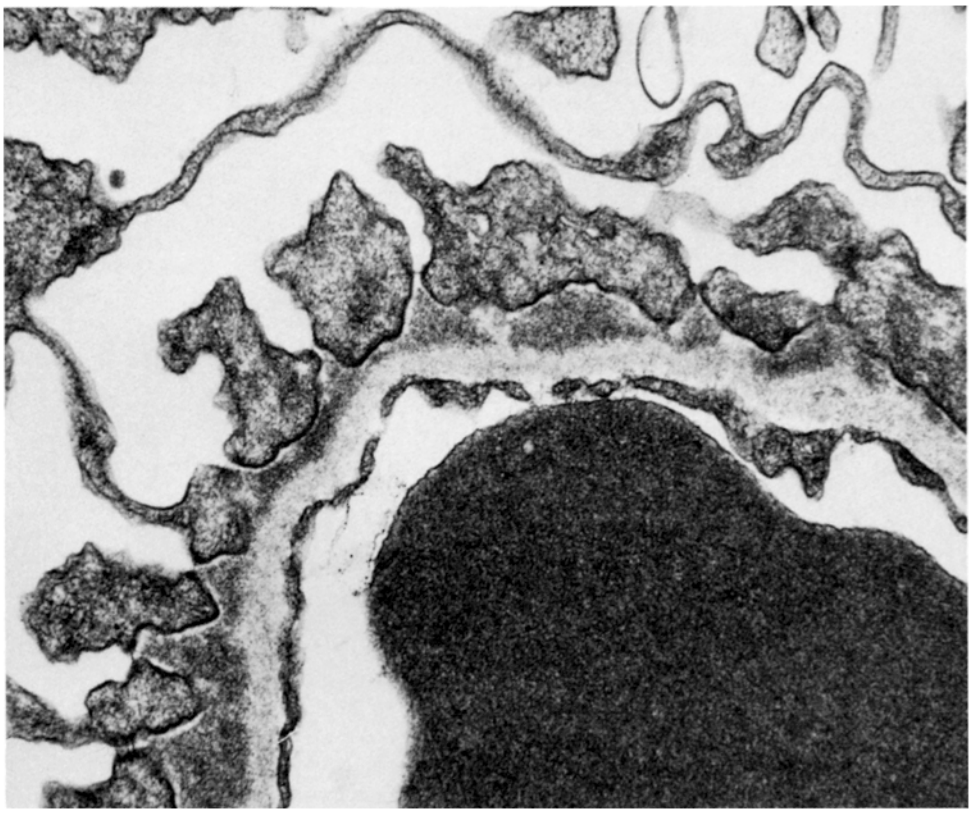

FIG. 5. The electron microscopic appearance of a glomerulus from a rat 3 d after perfusion of 20 μg cationized human IgG followed by intravenous injection of anti-human IgG. Massive subepithelial electron dense deposits are present (× 39,000).

of antibody. There was a remarkable difference between the removal rates of antigen in both groups. After perfusion with 20 μg of ^{125}I-labeled cationized human IgG, antigen disappeared from the kidney just as rapidly as in the case of 1.0 mg of antigen given intravenously. In contrast, a significant proportion of the cationized human IgG was removed more slowly from the perfused kidney when 2.5 mg of anti-human IgG antibody globulin had been given subsequently. 1.3 μg of cationized human IgG was still fixed to the perfused kidney on day 21. From the results shown in Fig. 6, it could be calculated that ~12 d were needed until one-half of the ^{125}I-labeled cationized human IgG, fixed on day 1, had been removed from the perfused kidney.

7. Disappearance Kinetics of ^{131}I-labeled Anti-Human IgG Antibody from the Kidney after In Situ Immune Complex Formation at Nephritogenic Levels. After *in situ* immune complex formation at nephritogenic levels (perfusion of 20 μg of cationized human IgG, intravenous injection of 2.5 mg ^{131}I-labeled anti-human IgG), the amount of anti-human IgG antibody in the perfused kidney remained almost unchanged during the first week. A more marked loss of antibody from the kidney was seen between days 7 and 14 (Fig. 7).

TABLE III

*Amount of Anti-Human IgG Injected to Induce Proteinuria**

Rabbit ^{131}I-anti-human IgG injected	Rabbit ^{131}I-anti-human IgG in left kidney	Rabbit ^{131}I-anti-human IgG in right kidney	Rabbit ^{131}I-anti-human IgG bound to left kidney (minus right kidney)	Rabbit ^{131}I-anti-human IgG in blood	Activity in right kidney in equivalents of blood contamination	Number exhibiting proteinuria >30 mg/d‡
mg	*μg*	*μg*	*μg*	*μg/ml*	*ml*	
1.0	15.0 ± 3.7§	1.8 ± 0.2	13.2 ± 1.8	22.5 ± 1.8	0.08	1/3
2.5	35.9 ± 5.0	4.0 ± 0.9	31.9 ± 5.7	44.9 ± 8.3	0.09	4/4
5.0	92.5 ± 17.7	9.2 ± 3.1	83.3 ± 19.1	104.4 ± 13.5	0.09	3/3
10.0	199.1 ± 24.3	20.8 ± 14.3	178.2 ± 24.2	221.3 ± 75.2	0.09	3/3
Control						
2.5‖	12.3 ± 1.9	8.6 ± 0.5	3.7 ± 1.9	66.3 ± 10.3	0.13	0/4
2.5 mg of normal rabbit IgG	8.6 ± 2.6¶	6.9 ± 0.3¶	2.3 ± 2.1¶	55.4 ± 5.6¶	0.12	0/3

* Rats were injected with anti-human IgG 1 h after renal perfusion with 20 μg cationized human IgG and killed on day 3, and uptake of ^{131}I-anti-human IgG antibody globulin was determined.
‡ Estimated on day 3.
§ Mean ± SD.
‖ Rats were perfused with 20 μg native human IgG.
¶ Amounts of ^{131}I normal rabbit IgG.

TABLE IV

Disappearance of Intravenously Injected Cationized Human IgG from the Kidney

Time	^{125}I-cationized human IgG bound*	
	Left kidney	Right kidney
h	*μg/kidney*	
1	21.1 ± 3.1‡	20.6 ± 13
3	13.9 ± 0.8	13.8 ± 16.2
6	6.9 ± 0.8	7.1 ± 0.7
12	3.7 ± 0.3	3.6 ± 0.2
24	1.6 ± 0.2	2.0 ± 0.2
48	1.2 ± 0.2	1.3 ± 0.1
72	1.1 ± 0.2	1.1 ± 0.2

* Rats were injected with 1 mg of ^{125}I-cationized human IgG through the tail vein.
‡ Mean ± SD of three rats.

Discussion

The idea that the deposition of circulating immune complex in the glomerular filtration barrier could be an initiating event in glomerulonephritis was unchallenged for many years. This consensus was derived from detailed analysis of acute and chronic serum sickness models (23, 24). In particular, subepithelial deposits were thought to be formed by this route in addition to the frequently occuring subendothelial and mesangial immune deposits. The earlier suggestion that serum sickness

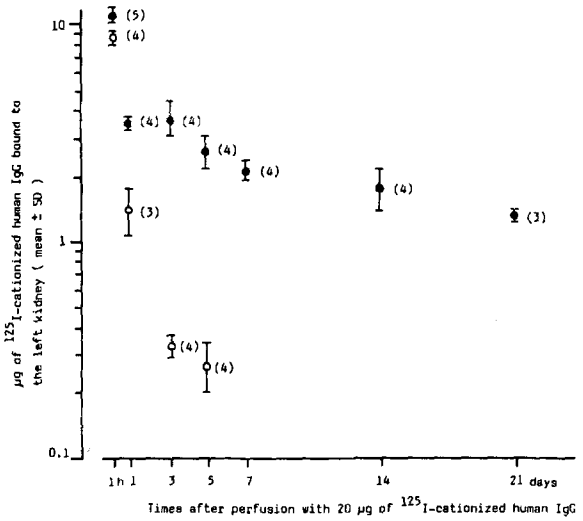

FIG. 6. The disappearance kinetics of cationized human IgG from the perfused kidney, with (●) and without (○) passive injection of antibody. Numbers in parenthesis denote number of animals studied at each time point.

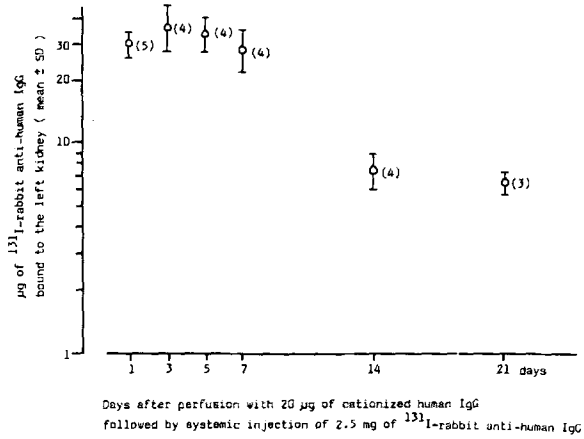

FIG. 7. Disappearance kinetics of anti-human IgG antibody from the *in situ* immune complex. Numbers in parenthesis denote number of animals studied at each time point.

nephritis was caused by *in situ* formation of immune complex (25) was more recently revived to explain observations relating to subepithelial deposit formation in Heymann (1, 2) and Lupus nephritis (26).

The probability of an *in situ* immune complex is more likely with antigens that can interact with structures of the GBM; cationic proteins fulfill this requirement (4–6, 15). An essential property of the GBM is the presence of discrete fixed anionic sites in the lamina rara externa and interna that convey an overall negative charge to the membrane (9, 10, 14). Previous studies (4) demonstrated that intravenously injected cationic moieties bound to the GBM if their molecular weight did not exceed a critical value lying between 500,000 and 900,000 and their pI was >8.5–9.5. Experiments employing the method of unilateral renal perfusion showed that cationized human

IgG and cationized horse spleen ferritin can act as planted antigens with which circulating antibody can react to form an *in situ* immune complex and induce massive proteinuria (5, 6). In this investigation, quantitative analysis of such a model was undertaken to measure the minimum quantities of antigen and antibody necessary for induction of kidney damage as well as to determine the fate of the complex. Cationized human IgG was selected because ferritin cannot be stably radioiodinated in our hands.

Cationized proteins localize throughout the body when injected intravenously, and under the conditions used here, ~4% of the cationized human IgG was found in the kidneys. This is explained by the high affinity of cationic substances for negative charges found throughout the vascular bed and on blood components, such as erythrocytes (17) or platelets (10). Unilateral renal perfusion via the left renal artery was used to preferentially localize the test material into the kidney. Provided that <100 μg of antigen was perfused, no antigen could be detected in the unperfused kidney by immunofluorescence. Up to 56% of the injected dose was found in the left kidney after 1 h, and at this time <1% still circulated. This effectively excludes the possibility that a significant deposition of circulating immune complexes could occur after passive administration of antibody. The absolute quantity of cationized human IgG deposited in the kidney increased with dose, although the percentage localizing fell from 50 μg downwards. We believe that saturation of the anionic sites in the GBM occurs and that additional antigen is localized elsewhere in the kidney; the finding of positive interstitial fluorescence only with higher doses supports this view. Administration of as little as 20 μg of high cationized human IgG (pI > 9.5) directly into the left renal artery and systemic injection of 2.5 mg of the IgG fraction of anti-human IgG 1 h later regularly induced a massive proteinuria in rats. This demonstrates clearly that only minimal amounts of antigen and antibody are necessary to cause overt kidney disease. At the time of antibody injection, 11.1 ± 0.8 μg of cationized human IgG was initially planted in the perfused left kidney, and there was only 0.07 μg in the unperfused right kidney (0.6% of the dose in the left kidney). At the onset of proteinuria, ~4 μg antigen and ~30 μg anti-human IgG antibody were present in the perfused kidney. It is interesting to compare the dose of antigen and antibody needed to induce renal damage in our model with those measured in other models of immunologic experimental glomerulonephritis. Relevant work concerning renal injury induced by passively given antibody against heterologous nephrotoxic gammaglobulin, which played the role of fixed antigen, was performed by Unanue and Dixon (27) in rats. They showed that 12–40 μg of kidney-fixing antibody (nephrotoxic antibody), which itself produced no proteinuria, had to be available as target antigen to induce proteinuria. In the model of one-shot serum sickness, a mean of 10 μg of bovine serum albumin (BSA) was deposited per kidney after elimination of BSA from the circulation (28). These values are of the same order as the quantity of cationic antigen required in our system.

When administered alone the majority of the cationized human IgG was removed from the kidneys within hours. Initially, immunofluorescence showed the antigen to be distributed in a virtually linear pattern along the GBM, reflecting the high density of antigen deposits. With time, the pattern became granular in nature and the intensity decreased, and by 24 h the glomeruli were mostly negative.

After binding of antibody, the removal of antigen from the kidney was retarded,

and 12 d was needed to remove one-half of the cationized human IgG. With the exception of the first 1 or 2 d, the immunofluorescent staining pattern for antigen and antibody was granular in nature. Deposition of rat C3 clearly preceded induction of proteinuria. We have not investigated whether complement is essential in causing tissue damage in this system. Rat IgG was first observed after 7 d, when the proteinuria was in decline and no second phase was seen, as usually occurs in nephrotoxic nephritis. This indicates that subsequent fixation of rat antibody to the immune deposits has little influence on the course of the disease. Electron microscopy revealed massive subepithelial dense deposit formation showing great similarity to findings in some human glomerulonephritides.

In our system (cationic antigen) and in one-shot serum sickness (28) (anionic antigen), the antigens were removed from the kidneys with a similar half-life of 10–14 d, indicating that the charge of the antigen had little influence on elimination from the complex. Cationized human IgG was removed faster from the kidney than the antibody, the binding of which remained almost unchanged during the first week. Perhaps the remaining circulating antibody continued to bind to deposited antigen or to the antigen-antibody complex in the kidney, or antigen was indeed removed faster than antibody from the immune complex. In addition, some antigen may be located in a part of the kidney not accessible to antibody and may therefore be eliminated independently.

A difference in the disappearance of antigen and antibody has been described by Fish et al. (29), who examined the course of one-shot serum sickness nephritis using immunofluorescence. However, it is not certain that a real difference exists here, because it was proposed (28) that the antigen may be masked but still be present. The half-life of kidney-fixed gamma globulin in nephrotoxic nephritis of the rat has been reported to be 52 d (30). A direct comparison of these results is of doubtful value since the mode of binding with the glomerular capillary walls is different.

Recent studies have provided evidence that *in situ* immune complex formation, which may be a cause of tissue injury in various organs (31, 32), can result in glomerular injury (1–6). In passive Heymans nephritis, it has been shown (1) that the GBM contains antigen similar to FX1A, found in the brush border of proximal tubular epithelial cells, either as intrinsic or planted antigen, which serves as a target for circulating antibody in the rat. Using heterologous antiserum, the total quantity of kidney-fixing antibody was shown to exceed 100 μg/kidney (33). Proteinuria did not usually reach a level of >100 mg/d from both kidneys (2, 33). In our study, proteinuria from a single kidney exceeded 100 mg/d after the deposition of ~30 μg of heterologous antibody before autologous rat IgG was detectable by immunofluorescence. Thus, there appear to be quantitative differences between our model and passive Heymans nephritis, although subepithelial dense deposits are prominent in both cases (2, 34). The glomerulus has other features that may promote *in situ* glomerular immune complex formation. Golbus and Wilson used the glycoprotein-binding property of the lectin concanavalin A (Con A) to plant it in the glomerular capillary wall. Systemic injection of anti-Con A antibodies resulted in *in situ* immune complex formation and proliferative glomerulonephritis (3). In this latter model the proteinuria induced lay within the range of 16–51 mg/d. The site of immune complex formation seems to be on the endothelial side of the GBM, because binding of Con A could be easily reduced by subsequent perfusion of alpha-methyl-mannoside. The

existence of complexes exclusively subendothelially may result in a less severe injury to the glomerular capillary wall. The mechanism of *in situ* complex formation is now seriously considered to operate in acute serum sickness nephritis, as experiments with isolated kidneys showed that alternating perfusion with BSA and anti-BSA resulted in subepithelial complex formation, whereas preformed immune complex failed to localize (35).

Recently, data were presented (36) concerning the relationship between glomerular permeability and the anionic glycosaminoglycan (GAG). In this work, the anionic component GAG in the lamina rara interna and externa was considered to be important in regulating the permeability of the glomerular capillary wall. The loss of these anionic sites following treatment with a specific enzyme, heparinase, remarkably enhanced both the penetration of native ferritin into the GBM and its excretion into the urinary space, whereas other enzymes such as hyaluronidase and chondroitinase ABC had no effect. The interaction of cationic antigen-antibody complex with the GBM could lead to a decrease or an alteration of the fixed anionic sites, resulting in a disturbance of glomerular permeability.

Summary

Cationized human IgG can bind to the rat glomerular basement membrane (GBM), act as planted antigen, and induce *in situ* immune complex formation accompanied by severe glomerulonephritis. Perfusion of highly cationized human IgG (isoelectric point > 9.5) via the left renal artery resulted in preferential localization within the perfused kidney (up to 56% of dose injected); after intravenous administration, only 4% was bound to the kidneys. The planted antigen was localized along the glomerular capillary walls and was accessible for antibody administered intravenously 1 h after perfusion, when virtually no antigen remained in the circulation.

Persistence of cationized human IgG in the perfused kidney was markedly prolonged when complexed with antibody; one-half the cationized human IgG was still present after 12 d. There was a difference in the disappearance rates of antigen and antibody, as cationized human IgG was removed faster from the kidney than the antibody, the binding of which remained almost unchanged during the first week. Renal perfusion of a minimum of 20 μg of cationized human IgG, followed by intravenous injection of antibody, regularly induced severe glomerulonephritis with a proteinuria of at least 100 mg/24 h. The degree and the persistence of proteinuria induced depended on the dose of cationized human IgG perfused.

Experiments using radiolabeled antigen and antibody showed that after renal perfusion of 20 μg cationized human IgG, 11.1 μg was kidney bound at the time of antibody injection. At the onset of proteinuria, 4.0 μg of antigen and 31.9 μg of anti-human IgG antibody were present in the perfused kidney. Immunofluorescence revealed immune deposits consisting of cationized human IgG and rabbit IgG (anti-human IgG) along the GBM. The staining pattern was linear (confluent) during the first 2 d and became granular during the course of the disease. Electronmicroscopically, a prominent finding was the accumulation of dense deposits, mainly in the subepithelial space and beneath the slit pores.

Received for publication 29 July 1981 and in revised form 19 October 1981.

References

1. Van Damme, B. J. C., G. J. Fleuren, W. W. Bakker, R. L. Vernier, and P. J. Hoedemaeker. 1978. Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab. Invest.* **38:**502.
2. Couser, W. G., D. R. Steinmuller, M. M. Stilmant, D. J. Salant, and L. M. Lowenstein. 1978. Experimental glomerulonephritis in the isolated perfused rat kidney. *J. Clin. Invest.* **62:**1275.
3. Golbus, S. M., and C. B. Wilson. 1979. Experimental glomerulonephritis induced by *in situ* formation of immune complexes in glomerular capillary wall. *Kidney Int.* **16:**148.
4. Vogt, A., U. Schmidt, H. Takamiya, and S. R. Batsford. 1980. *In situ* immune complex nephritis and basic proteins. *In* Proceedings of European Dialysis and Transplant Association. Pitman Medical Publishing Co., Ltd., Tunbridge Wells, England. **17:**613.
5. Batsford, S. R., H. Takamiya, and A. Vogt. 1980. A model of in situ immune complex glomerulonephritis in the rat employing cationized ferritin. *Clin. Nephrol.* **14:**211.
6. Batsford, S. R., T. Oite, H. Takamiya, and A. Vogt. 1980. Anionic binding sites in the glomerular basement membrane: possible role in the pathogenesis of immune complex glomerulonephritis. *Renal Physiol.* **3:**336.
7. Kefalides, N. A., and R. J. Winzler. 1966. The chemistry of glomerular basement membrane and its relation to collagen. *Biochemistry.* **5:**702.
8. Spiro, R. G. 1976. Studies of the renal glomerular basement membrane. *J. Cell. Biol. Chem.* **242:**1915.
9. Kanwar, Y. S., and M. G. Farquhar. 1979. Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. U. S. A.* **76:**1303.
10. Kanwar, Y. S., and M. G. Farquhar. 1979. Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the lamina rarae by cationic probes. *J. Cell. Biol.* **81:**137.
11. Jones, D. B. 1969. Mucosubstances of the glomerulus. *Lab. Invest.* **21:**119.
12. Latta, H., W. H. Johnston, and T. M. Stanley. 1975. Sialoglycoproteins and filtration barriers in the glomerular capillary wall. *J. Ultrastruct. Res.* **51:**354.
13. Latta, H., and W. H. Johnston. 1976. The glycoprotein inner layer of glomerular capillary basement membrane as a filtration barrier. *J. Ultrastruct. Res.* **57:**65.
14. Caulfield, J. P., and M. G. Farquhar. 1976. Distribution of anionic sites in glomerular basement membranes: their possible role in filtration and attachment. *Proc. Natl. Acad. Sci. U. S. A.* **73:**1640.
15. Rennke, H. G., R. S. Cotran, and M. A. Venkatachalam. 1975. Role of molecular charge in glomerular permeability. Tracer studies with cationized ferritin. *J. Cell. Biol.* **67:**638.
16. Brenner, B. M., M. P. Bohrer, C. Baylis, and W. M. Deen. 1977. Determinants of glomerular permselectivity: insights derived from observations in vivo. *Kidney Int.* **12:**229.
17. Danon, D., L. Goldstein, Y. Marikovsky, and E. Skutelsky. 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. *J. Ultrastruct. Res.* **38:**500.
18. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (Lond.).* **195:**281.
19. Minden, P., and R. S. Farr. 1973. Vol. 1, chapter 15. The ammonium sulphate method to measure antigen binding capacity. *In* Handbook of Experimental Immunology, D. M. Weir, editor. Blackwell Scientific Publications, Oxford.
20. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy. Appl. Immunol.* **29:**185.
21. Weichselbaum, T. E. 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Amer. J. Clin. Pathol.* **10:**40.
22. Renal Biopsy Management and Processing by the Pathologist. Chapter 3, p. 8 in 'Renal

Pathology in Biopsy', Zollinger, H. U., and M. J. Mihatsch, editors. Springer Verlag, Heidelberg.

23. Germuth, F. G., Jr., and E. Rodriguez. 1973. Immunopathology of the Renal Glomerulus: Immune Complex Deposit and Anti-basement Membrane Disease. Little Brown and Company, Boston. chapter 2, p. 15.
24. Wilson, C. B., and F. J. Dixon. 1974. Diagnosis of immunopathologic renal disease. *Kidney Int.* **8:**389.
25. Hawn, C. V., and C. A. Janeway. 1947. Histological and serological sequences in experimental hypersensitivity. *J. Exp. Med.* **85:**571.
26. Izui, S., P. H. Lambert, and P. A. Miescher. 1976. In vitro demonstration of a particular affinity of glomerular basement membrane and collagen for DNA: a possible basis for local formation of DNA-anti-DNA complexes in systemic lupus erythematosus. *J. Exp. Med.* **144:** 428.
27. Unanue, E. R., and F. J. Dixon. 1965. Experimental glomerulonephritis. VI. The autologous phase of nephrotoxic nephritis. *J. Exp. Med.* **121:**715.
28. Wilson, C. B., and F. J. Dixon. 1970. Antigen quantitation in experimental immune complex glomerulonephritis. I. Acute serum sickness. *J. Immunol.* **105:**279.
29. Fish, A. J., A. F. Michael, R. L. Vernier, and R. A. Good. 1966. Acute serum sickness nephritis in the rabbit. An immune deposit disease. *Amer. J. Pathol.* **49:**997.
30. Unanue, E. R. and F. J. Dixon. 1965. Experimental glomerulonephritis. V. Studies on the interaction of nephrotoxic antibodies with tissues of the rat. *J. Exp. Med.* **121:**697.
31. Clagett, J. A., C. G. Wilson, and W. O. Weigle. 1974. Interstitial immune complex thyroiditis in mice: The role of autoantibody to thyroglobulin. *J. Exp. Med.* **140:**1439.
32. Bigazzi, P. E., L. L. Kosuda, K. C. Nsu, and G. A. Andres. 1976. Immune complex orchitis in vasectomized rabbits. *J. Exp. Med.* **143:**382.
33. Salant, D. J., C. Darby, and W. G. Couser. 1980. Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J. Clin. Invest.* **66:**71.
34. Salant, D. J., S. Belok, M. M. Stilmant, C. Darby, and W. G. Couser. 1979. Determinations of glomerular localization of subepithelial immune deposits. Effects of altered antigen to antibody ratio, steroids, vasoactive amine antagonists and aminonucleoside of puromycin on passive Heymann nephritis in rats. *Lab. Invest.* **41:**89.
35. Fleuren, G., J. Grond and P. J. Hoedemaeker. 1980. *In situ* formation of subepithelial glomerular immune complexes in passive serum sickness. *Kidney Int.* **17:**631.
36. Kanwar, Y. S., A. Linker, and M. G. Farquhar. 1980. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Cell. Biol.* **86:**688.