EXPERIMENTAL GLOMERULONEPHRITIS

V. Studies on the Interaction of Nephrotoxic Antibodies with Tissues of the Rat*, ‡

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(Received for publication, January 7, 1965)

Much of our information and prejudice regarding the action of host autoantibodies to tissue-fixed antigens is based on experience with heterologous antitissue antibodies, particularly antikidney antibodies. The interaction of heterologous antikidney antibodies with the renal antigens, largely in the glomerular capillary walls, and with antigens of other vascular tissues such as lung, adrenal, and placenta has been the subject of numerous reports (1–4). The rapid clearance of these antibodies from the circulation and their fixation to tissues has been determined (5–7). The pathologic consequences of this antigen-antibody reaction and some of the mediators of the injury are also recognized (8, 9). However, whether some autologous antitissue antibodies might have capabilities for inducing tissue injury similar to those of heterologous antibodies is still not certain. Since quantitative studies of autoantibodies are virtually impossible because of ever present antigens capable of removing antibody from the circulation, critical observations on the autoantibody-tissue antigen interaction are difficult. Even in cases of well recognized antitissue antibody responses, such as exists in experimental allergic encephalomyelitis (10, 11), it is not possible to determine which, if any, of the several types of antibrain antibodies in the circulation may be reacting with brain antigens. Thus, for the present it appears that we will have to continue to rely on observations based on the use of heterologous antibodies to expand our knowledge of the pathogenetic potentialities of antitissue antibodies.

The present studies were intended to further define the action of nephrotoxic antibody (NTAb) quantitating, insofar as possible, the interaction of various kinds of NTAb, duck and rabbit, gamma-2 globulin (γ_2) and gamma-1 macroglobulin (γ_{1M}), with tissue antigens of the rat and the resultant tissue injury. It has been possible to determine the number of antibody molecules fixing per unit area of glomerular capillary surface which will induce immediate functional and morphologic abnormalities and the number which will saturate available

* This is publication number 98 from the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla.

‡ This study was supported by United States Public Health Service grants AI 03897 and AEC Contract AT(04-3)-410.

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antigenic sites in the kidneys. From these data some approximation of the anatomical mass of immunologic reactants can be made. The rates of fixation of the various antibodies to, and their loss from, the various tissues have also been determined. A comparison of the behavior and relative pathogenicity of γ_{2}- and γ_{1M}-NTAb has also been possible. These observations provide quantitative data which not only describe the early events in nephrotoxic serum nephritis (NTN) but will perhaps also be applicable to antitissue antibody-antigen reactions in general.

Materials and Methods

Rabbit and duck NTAbs produced by immunization with whole rat kidney were used in these experiments (12). The rabbits were immunized by the intraperitoneal route with the kidney antigen suspended in 0.15 M NaCl solution; the ducks were immunized by intramuscular injection with the antigen in complete Freund's adjuvant. Two rabbit nephrotoxic sera (NTS) were obtained 1 week after a single intraperitoneal injection of 300 mg of kidney antigen and will be referred to as primary NTAb. All other NTS were obtained after 6 months to 2 years of repeated immunizations. Recipients of the NTAb were outbred strains of Sprague-Dawley rats varying in weight from 150 to 180 gm. In all the experiments nephritis was induced by a single intravenous injection of NTAb. Urine was collected during 24-hour periods and total protein determined as previously reported (12). Normal rats of this strain and weight excrete an average of 2.7 mg of protein in a 24-hour period; rats of 200 to 400 gm excrete an average of 15.6 mg. In all the experiments proteinuria over 20.0 mg/24 hours was considered abnormal.

Fluorescent antibody studies employed the techniques and antisera previously reported (12). Fluorescent antisera for rat gamma globulin, rat beta-1C (β1C-) globulin, rat fibrinogen, rabbit gamma globulin, and duck gamma globulin were used.

Five experiments were undertaken as follows:

I. Characterization of NTAb.—Rabbit and duck NTS were separated on a sucrose density gradient using the technique reported by Kunkel (13). Fractions were collected by puncture of the bottom of the tube and proteins identified by microimmunoelectrophoresis using sheep anti-rabbit gamma globulin and rabbit anti-duck gamma globulin (12). Total protein in these fractions was determined by the Folin Ciocalteu method (14). NTAb activity was determined by the amount of I^{131}-labeled antibody which fixed *in vivo* to the kidneys. Fractions containing identical proteins were pooled from several gradient runs, dialyzed against 0.15 M NaCl overnight, concentrated by pressure dialysis, and then injected into rats to determine their nephritogenic properties. The different fractions were also studied after treatment with mercaptoethanol; they were placed in a dialysis bag, and dialyzed against 0.1 M mercaptoethanol for 12 to 24 hours at room temperature and then against 0.01 M iodoacetamide for 24 hours in the cold; these treated fractions were tested for their nephritogenic activity as well as for their ability to fix to the kidney when labeled with I^{131}.

Duck nephrotoxic proteins were also collected from a sephadex G-200 column using a tris buffer at 8.2 pH. Duck nephrotoxic gamma globulin (NTGG) was obtained by preparative electrophoresis using pevikon as a supportive media. Analytical ultracentrifuge studies were performed on two preparations of duck NTGG. The first was duck gamma globulin as obtained by preparative electrophoresis and contained only the three immunoglobulins that will be further described; the second was obtained by ammonium sulfate fractionation at a 40 per cent concentration and contained in addition to the immunoglobulins, traces of hemoglobin and other beta globulins. Analytical ultracentrifugation was performed in a Beckman model

E centrifuge operated at 59,780 RPM. Photographic plates were measured with a two dimensional microcomparitor. Sedimentation coefficients at infinite dilution were calculated by the method of least squares from runs at 3 protein concentrations for each preparation. The partial specific volume of the proteins was assumed to be 0.73.

II. Quantitation of the Kidney-Fixing Antibody (KFAb) in Various NTS and its Nephritogenic Potency.—The amount of NTAb in each NTS was determined by a method similar to the one described by Pressman (1). In essence, the gamma globulin fraction from each serum was labeled with I^{131} and injected into rats which were then sacrificed and their perfused kidneys counted for radioactivity. For the rabbit NTS, only the γ_2-globulin fraction was obtained; this was done by column chromatography using diethylaminoethyl cellulose with a phosphate buffer of 0.0175 M at a pH of 6.4. For the duck NTS, gamma globulins were obtained by preparative electrophoresis using pevikon as a supportive media; the duck gamma globulin preparations contained principally the light sedimenting gamma globulins with traces of γ_{1M}-globulins. Labeling with I^{131} was carried out by the chloramine T method as modified in this laboratory (15). The different preparations had a specific activity of 2 to 15 μc per mg of gamma globulin. Each labeled preparation was injected into groups of 3 to 5 rats which were sacrificed 3 days later. Each rat usually received 500 to 800 μg of labeled gamma globulin. At sacrifice, the kidneys of each rat were perfused *in situ via* the abdominal aorta with 75 ml of 0.15 M NaCl. Blood was also obtained from each animal at sacrifice. The kidneys and 1.0 ml samples of blood were counted in a sodium iodide crystal scintillation counter. The radioactive counts were corrected for background and decay of I^{131}. There was a maximum variation of 8 to 12 per cent in kidney counts among rats of each group. The amount of KFAb was expressed as the average of each group of rats. If these kidneys, obtained 3 days postinjection, are homogenized in a blendor and centrifuged at 1500 *g* for 1 hour, 85 to 90 per cent of the I^{131} NTGG is found fixed to the sedimentable, insoluble fraction. To determine if the radioactivity found in the soluble fraction of kidney (10 per cent) could be contamination with intravascular blood remaining after perfusion or labeled antibody complexed to soluble renal antigens two further control experiments were made. In the first experiment the kidneys, obtained 3 days after labeled NTGG injection, were homogenized and the soluble fractions obtained. The soluble fractions were concentrated by pressure dialysis and submitted to sucrose density gradient ultracentrifugation using the same technique as reported before; as control the native I^{131}-labeled NTGG and I^{131} normal rabbit gamma globulins (obtained from Pentex Inc., Kankakee, Illinois, Lot 33) were also run. All three preparations showed a single large peak of radioactivity corresponding to the γ_2-globulin; the soluble fraction of the kidney homogenate, however, also showed a faster sedimenting material which comprised 7 to 12 per cent of the total radioactivity of the soluble fraction and which was interpreted as a possible complex of NTAb with soluble renal antigens. In the second experiment normal rabbit gamma globulin was labeled with I^{131}, and injected into rats which were then studied as described above. This experiment showed that after perfusion each pair of kidneys was contaminated with the amount of radioactivity present in 0.09 to 0.17 ml of blood. These kidneys when fractionated into sediment and soluble fractions showed 5 per cent of the radioactivity in the sediment and the remaining in the soluble fraction. These two control experiments demonstrated that the radioactivity found in the soluble fraction of kidney was largely due to contamination with remaining intravascular blood and that at the most 10 per cent of it (1 per cent of the total radioactivity in the kidney) could be attributed to antibody bound to soluble renal antigens.

Two approaches were taken in order to quantitate the nephritogenic potency of various KFAbs: (*a*) Different rabbit and duck NTS were injected in amounts of 1.0 ml into groups of 4 to 6 rats and proteinuria determined on 24-hour collection of urine on days 1, 3, 10, and 35 after injection. The amount of KFAb in 1 ml of each serum was then calculated using the

percent of I^{131} gamma globulin in each serum which localized in the kidneys and multiplying this by the total gamma globulin of each ml of that serum. The total gamma globulin of each serum was determined by obtaining the percentage of gamma globulin by paper electrophoresis (spinco electrophoresis set with a Durrum vertical cell) and multiplying this by the total protein of the serum. The renal damage as represented by proteinuria and histopathology was correlated with the amount of KFAb. (*b*) The amounts of KFAb in various preparations of rabbit and duck NTGG were determined. Different calculated amounts of KFAb were then injected into groups of 3 to 5 rats and 24-hour urine collections and histologic examinations done as in group (*a*).

III. Rate of Fixation of NTAb.—Experiments were done to determine the rate of fixation of rabbit and duck NTAb to the kidneys, liver, and lungs of the rat as well as the rate of removal of these antibodies from the circulation. For this purpose NTGG from rabbit and duck were labeled with I^{131} and injected into rats; groups of 3 rats were sacrificed at 10 and 30 minutes, 1, 6, and 12 hours, 1 and 4 days after injection. Each rat was exsanguinated and all organs perfused with 0.15 M NaCl. Kidneys, liver, and lung were homogenized and fractionated in sedimentable and soluble fractions as described above. The sedimentable fraction was washed 5 times with 0.15 M NaCl and the amount of radioactivity in the washed sediment was then determined. To determine residual kidney-fixable antibody in the circulation, the sera from rats sacrificed at each time interval were pooled and reinjected into 2 rats; these were then sacrificed 3 days later and the percentage of injected radioactivity in the sedimentable fraction of the kidney was determined. Thus, for each time interval after injection of the NTGG the per cent of antibody fixed to the kidney, liver, and lung was measured. In addition, that portion of the *circulating* NTGG which was capable of fixing in the kidney was also found. The total KFAb detectable at each time interval was considered to be the sum of the antibodies fixed in the kidneys of the original recipient plus that remaining in the circulation which was capable of fixing to the kidneys of the 2nd animal.

A small amount of KFAb was found free in the circulation 1 hour after injection of labeled NTGG and experiments were done in order to compare this antibody with that which fixed promptly to the kidney. Three characteristics of this antibody were investigated: (*a*) its rate of fixation to the kidney; (*b*) its site of fixation, *i.e.* kidney's sediment or soluble fractions; and (*c*) its half-time in the kidney. Three rats were injected with I^{131}-labeled rabbit NTGG and sacrificed 1 hour later; the sera were pooled and reinjected into 12 rats that were sacrificed in groups of 3, at 10 minutes and 3, 12, and 21 days after injection. Radioactivity was determined on the whole kidneys and in the sedimentable and insoluble fractions. The whole labeled NTGG was also injected into 12 rats which were sacrificed and studied at the same time intervals.

IV. Persistence of NTAb in Different Organs.—The persistence of NTAb was determined in kidneys, adrenals, liver, and lungs. For this purpose different preparations of rabbit and duck NTGG were labeled with I^{131} and each injected into groups of 15 to 18 rats. Rabbit NTGG was obtained as described before, duck NTGG was obtained by preparative electrophoresis and contained primarily γ_{2A}- and γ_{2B}-globulins with a trace of γ_{1M}; duck NT γ_{1M} was obtained by sephadex G-200 column as described before and was reasonably pure. Groups of 3 rats were sacrificed weekly beginning 3 days after injection and the radioactivity determined in each organ after perfusion with 0.15 M NaCl. Control groups were injected with normal I^{131}-labeled rabbit and duck gamma globulins and treated similarly. Disappearance rates were calculated from semi logarithmic plots with the percentage of the injected material bound to the organ on the ordinate and the days postinjection in the abscissa (Fig. 4). The term half-disappearance time will be employed in these studies despite the fact that presumably a small amount of circulating antibody fixes continuously to the tissues.

V. Amounts of Available Renal Antigen.—Experiments were made with rabbit and duck

NTGG to determine the amount of KFAb needed to saturate the available antigenic sites of the kidney. Groups of rats were injected simultaneously with a trace amount of 500 to 800 μg I^{131}-labeled NTGG and varying amounts of the same unlabeled NTGG; 3 to 5 rats were injected for each dose of material. All rats were sacrificed 1 week after injection and the kidneys counted for radioactivity after perfusion. Each experiment included a group of rats where only the trace amount of labeled NTGG was injected. At these trace concentrations the percentage of KFAb is directly proportional to the amount of NTGG injected (16). The total amount of KFAb injected at each dose of NTGG was then calculated from this percentage. The amount of KFAb bound in the kidney at each concentration was calculated by multiplying the total gamma globulin injected by the percentage of I^{131}-labeled material found in the kidney.

RESULTS

1. Characterization of NTAb.—Rabbit KFAb was localized exclusively in the γ_2-globulin fraction of all the long-term immunized animals. The two primary KFAbs showed antibody activity in both the light and heavy fractions of the sucrose density gradient. However, the total amount of KFAb of these preparations was too small to cause proteinuria (10 and 17 μg of KFAb per ml of serum). The heavy gamma fraction of the sucrose density gradient contained 0.03 and 0.06 per cent of KFAb. The light gamma globulin fraction contained 0.09 and 0.15 per cent of KFAb. No reduction in the ability of I^{131}-labeled heavy or light rabbit antibody to fix to the kidneys was found after treatment with mercaptoethanol.

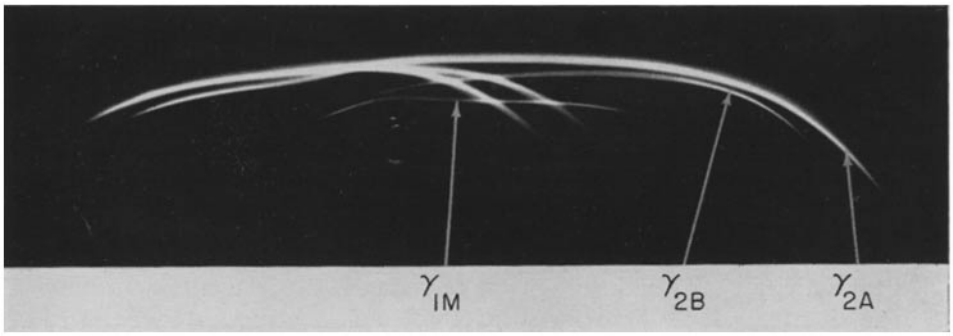

FIG. 1. Immunoelectrophoresis of a crude globulin fraction of duck serum; in the trough was placed a rabbit anti-duck globulin serum. Arrows point to γ_{2A}-, γ_{2B}-, and γ_{1M}-globulins. Two other unidentified globulins are noted in the beta area.

Duck KFAb activity was localized in three different immunoglobulins. The immunoelectrophoretic pattern of duck NTGG is illustrated in Fig. 1. Analytical ultracentrifuge studies were made on two preparations of duck NTGG. The preparation obtained by ammonium sulfate fractionation yielded 3 peaks with $s_{20,w}$ values of 5.73, 7.36, and 17.25; their relative concentrations were 54.2, 31.7, and 7.6 per cent with 6.5 per cent of a lighter sedimenting material.

The preparation obtained by preparative electrophoresis yielded 3 peaks with $s_{20,w}$ values of 5.89, 7.43, and 16.93; their relative concentrations were 65.6, 30.8, and 13.6 per cent. We have tentatively named the 5.8S protein as γ_{2A} and the 7.4S protein as γ_{2B}. No separation of γ_{2A} and γ_{2B} was obtained by preparative electrophoresis and only partial separation by sucrose density gradient or sephadex G-200 fractionation. A representative density gradient is shown in Fig. 2. Aliquots P plus 1, 5, and 9 which contained γ_{1M}, γ_{2B}, and γ_{2A} respectively, were labeled with I^{131} to determine their content of KFAb. Anti-

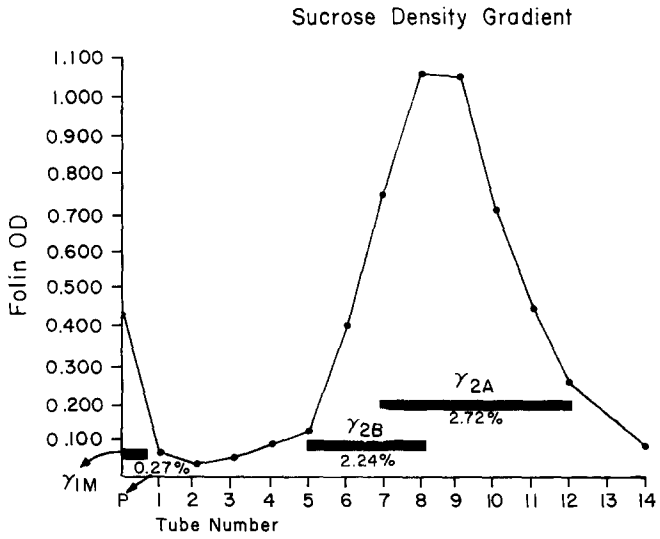

FIG. 2. Sucrose density gradient of duck NTS. Identification of each protein was made by immunoelectrophoresis. The percentages represent the amount of KFAb in each fraction examined (P plus 1, 5, and 9). The ordinates represent protein concentration of each fraction; abscissa represents tube numbers; P represents the pellet.

body activity was found in all of them as evidenced by fixation to the kidney of 0.28, 2.12, and 2.20 per cent respectively. These same fractions were treated with mercaptoethanol and tested for their KFAb content; no reduction in their ability to fix to the kidney was noted. Comparable results were obtained on fractions obtained by sephadex G-200 fractionation. A preparation containing both γ_{2A} and γ_{2B} was tested for its biologic activity by injecting into rats and testing for proteinuria. In 2 rats tested an average proteinuria of 197 mg was obtained 24 hours after injection (corresponding to the proteinuria induced by 250 to 350 μg KFAb). The same preparation was treated with mercaptoethanol and tested for its biologiacl activity in the same way; an average proteinuria in 2 rats of 47.6 mg was obtained 24 hours after injection (proteinuria corresponding to that caused by 150 to 200 μg of untreated KFAb). The γ_{1M}-

NTAb was also tested for biologic activity; proteinuria was obtained with injections of 27.2 μg or more of KFAb. 15 μg KFAb produced no proteinuria. This biological activity was completely abolished by treating the preparation with mercaptoethanol.

II. Quantitation of the KFAb in Various NTS and its Nephritogenic Potency. —Table I shows the results of studies intended to relate KFAb and nephrotoxicity of different preparations of rabbit NTS or NTGG. As noted in Table I, all rats developing an immediate proteinuria of 100 mg or more per 24 hours

TABLE I

Quantitative Studies with Rabbit NTS or NTGG

Rabbit No.	Injection	KFAb.	Proteinuria			
			Day 1	Day 3	Day 10	Day 35
		μg	*mg/24 hrs.**	*mg/24 hrs.**	*mg/24 hrs.**	*mg/24 hrs.**
1	Serum	310	121	90	212	188
2	Serum (3.5 ml)	297	136	115	148	111
3	Serum	251	147	98	156	168
4	Serum (1.5 ml)	224	103	87	85	104
5	Serum	206	158	176	269	182
4	Serum (1 ml)	153	9	16	52	48
6	Serum	86	17	3	22	23
2	Serum (1 ml)	85	13	7	18	26
7	Serum	47	7	6	22	23
8	Gamma globulin	100	11	5	11	16
	Gamma globulin	150	37	17	16	14
	Gamma globulin	175	64	76	48	91
	Gamma globulin	200	52	94	80	98
	Gamma globulin	250	202	261	169	162

* Each figure is average of 4 rats.

received more than 200 μg of KFAb. Some rats receiving approximately 150 μg KFAb and all rats receiving 175 to 200 μg of KFAb developed a moderate proteinuria during days 1 and 3; rats receiving less than 150 μg of KFAb did not develop significant proteinuria. 200 μg KFAb produces a maximum proteinuria and greater amounts did not cause significantly greater proteinuria. Immediate proteinuria could be obtained with any NTS if the amount of KFAb injected was adequate. Thus rabbit NTS 2 and 4 which were relatively weak and contained inadequate amounts of KFAb in 1 ml of serum to induce proteinuria when injected in 1.5 and 3.5 ml volumes caused immediate proteinuria. Thus no significant qualitative difference was found among the different preparations examined. Histologic and fluorescent antibody studies were done on rats sacrificed on days 3 and 35. Proliferative lesions in the glo-

meruli as noted by 10 to 15 per cent increases in the number of fixed glomerular cells were noted in all groups receiving 150 μg or more of KFAb; however, alterations in the glomerular basement membrane as evidenced by thickening and increase in PAS-positive material was seen only in rats receiving 200 μg or more. Fluorescent antibody studies made 3 days after injection showed rabbit gamma globulin in a membranous pattern in all the groups (see reference 12 for description of patterns of fluorescence); rat β1C-globulin was positive in a membranous pattern in all groups receiving 80 μg or more of rabbit KFAb.

TABLE II

Quantitative Studies with Duck NTAb

Duck	KFAb. Injected	Proteinuria		
		Day 1	Day 3	Day 14
	μg	*mg/24 hrs.*	*mg/24 hrs.*	*mg/24 hrs.*
15	224 (1.0 ml)	130.0	36.0	31.0
	448 (2.0 ml)	223.0	121.0	260.0
12	120 (1.0 ml)	1.0	0.7	5.0
	240 (2.0 ml)	32.0	16.0	53.5
17	120	5.4	0.7	7.2
	150	58.4	1.6	14.7
	180	160.8	3.1	10.2
	200	154.0	3.1	12.1
	250	274.0	50.6	27.0
	300	376.6	93.6	86.0
	350	438.3	120.0	114.7
	400	331.6	290.6	163.5

* Each figure is average of four rats.

At this time rat gamma globulin and rat fibrinogen were regularly seen in the glomeruli distributed in a mesangial pattern only.

Table II shows the results of studies employing different preparations of duck NTGG. Again, a direct correlation was present between amounts of KFAb injected and degree of acute proteinuria. Proteinuria was immediate but very transitory in rats receiving 150 to 200 μg of KFAb. However, beyond 200 μg, proteinuria was immediate and continuous throughout 2 weeks of observation in most rats. With any given duck NTGG (as No. 12 in Table II) acute proteinuria could be obtained if the amount of KFAb was sufficient. Histologic and fluorescent antibody studies were done on rats sacrificed on day 3 and 15. Proliferative changes were evident in groups receiving 250 μg or more of KFAb; basement membrane alterations were likewise noted but mainly at doses of

350 μg or more. Fluorescent antibody studies done on rats sacrificed 3 days after injection showed duck gamma globulin in a membranous pattern in all the groups. At this time rat β1C-globulin, gamma globulin, and fibrinogen were noted only in a mesangial pattern of distribution.

III. Rate of Fixation of NTAb.—Experiments were performed with 3 different preparations of rabbit NTGG and 2 of duck NTGG. A representative experiment is shown in Table III. In all the experiments irrespective of the preparation, 65 to 89 per cent of the total amount of antibody found fixed to the kidney at 1 hour was already bound during the first 10 minutes after injection. The maximum amount of kidney-fixed antibody was found 1 hour after injection following which there was a gradual loss. Results were different in the liver and lung. The maximum fixation was obtained 10 minutes after injection; after this period there followed a relatively rapid decrease in the amounts of fixed antibody for the next 1 to 6 hours with a subsequent slow rate of decay. In the experiment of Table III 57.9 per cent of the rabbit NTAb fixed in the liver at 10 minutes was removed within the first 6 hours; 66.6 per cent of the

TABLE III

Rate of Fixation of NTAb

Time after injection	Injected NTGG fixed in the sedimentable fractions of:			Circulating NTGG capable of fixing to sediment of kidney	KFAb. total remaining in circulation
	Kidneys	Liver	Lungs		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
			Duck NTGG		
10 min.	0.56	3.11	0.25	0.14	20.0
30 min.	0.83	2.36	0.23	0.18	17.8
1 hr.	0.85	2.40	0.15	0.16	15.8
6 hrs.	0.79	2.20	0.13	0.09	10.2
1 day	0.78	1.34	0.09	0.05	6.0
4 days	0.79	0.95	0.05	—	—
			Rabbit NTGG		
10 min.	0.62	2.63	0.15	0.15	19.4
30 min.	0.61	1.59	0.09	0.09	14.7
1 hr.	0.73	1.51	0.08	0.08	9.9
6 hrs.	0.71	1.11	0.05	0.06	7.7
1 day	0.71	1.08	0.07	0.04	5.3
4 days	0.69	0.62	0.04	—	—

The figures in columns 2 to 5 represent the percentage of injected material which fixes to the sediment of the entire organ. Total KFAb. was calculated by adding the figures of columns 2 and 5. All results are average of 3 to 5 rats.

rabbit NTAb fixed in the lung at 10 minutes was removed within a similar period. In the same experiment, 29.3 per cent of the duck NTAb fixed in the liver at 10 minutes was removed within the first 6 hours; 48.0 per cent of the duck NTAb fixed in the lung at 10 minutes was removed within a similar period. The remaining antibody showed half-disappearance times comparable to those seen in Table IV.

In all the experiments KFAb was found in the circulation during the first 24-hour period following injection of NTGG (Fig. 3). Rabbit NTGG showed approximately 80 per cent of the total KFAb removed from the circulation within 10 minutes and the last 10 per cent was removed with a half-time of 25.0 hours. Twenty-four hours after injection of rabbit NTGG 5.3 per cent of

TABLE IV

Persistence of Rabbit and Duck NTAb in Organs of the Rat

NTAb	Half-disappearance times			
	Kidney	Liver	Lung	Adrenal
	days	*days*	*days*	*days*
Rabbit NTAb. (7)	52.5	7.4	10.0	10.9
Duck NTAb.-γ_{2A}, γ_{2B} (2)	58.6	17.5	5.0	59.0
Duck NTAb.-γ_{1M} (2)	7.3	—	—	—

Figures in parentheses indicate number of antibody preparations studied.

the total KFAb was still present in the circulation. Eighty-one per cent of the total KFAb of duck NTGG was removed from the circulation within 10 minutes and the last 17 per cent with a half-time of 24.0 hours. Twenty-four hours after injection of duck NTGG, 6.0 per cent of the total KFAb was present in the circulation.

The KFAb remaining in the circulation 1 hour after injection was compared to the whole NTAb in several ways. First, this residual circulating KFAb, after transfer to a normal rat, localized in the sedimentable part of the kidney homogenate as did the whole NTAb. Second, the rate of fixation in the kidney of this antibody was much slower than the whole NTAb, 39.5 per cent of the total KFAb bound in the kidney fixed in the first 10 minutes *vs.* 90 per cent. Finally, the half-disappearance time of this residual antibody showed two components; 52 per cent of the antibody had a half-time of 22 days in the kidney while 48 per cent had a 75 day half-time identical with that found for the whole NTAb.

IV. Persistence of NTAb in Different Organs.—The average half-disappearance time of the different preparations of rabbit and duck NTGG in the various tissues are shown in Table IV. An experiment with a representative rabbit NTAb is shown in Fig. 4. Rabbit NTAb had a long half-time in the kidney as

measured after day 10 varying from 42.0 to 69.0 days. The greater apparent rate of loss from the kidney seen from 3 to 10 days after injection may be in part influenced by contamination of circulating labeled gamma globulin and in part by the presence of some short-lived antibodies. In the different preparations no correlation was found between the content of KFAb and the half-time of the antibody in the kidney. The two primary NTAbs were also studied for persistence in the kidneys (not included in Table IV) and showed half-times

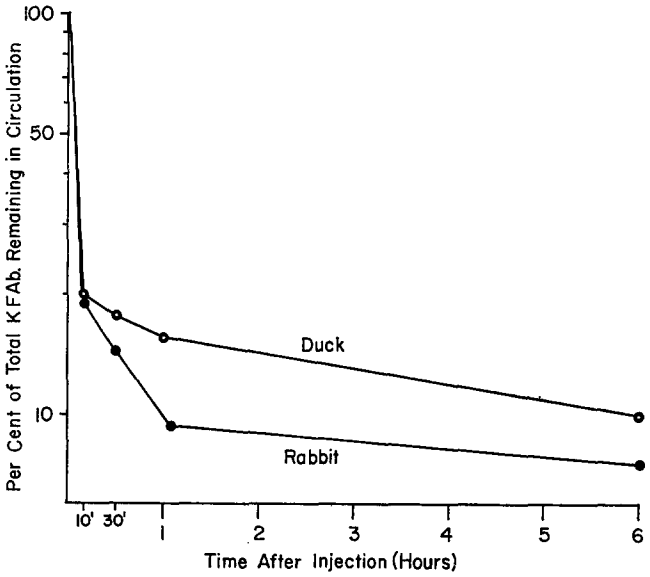

FIG. 3. Rate of disappearance from the circulation of KFAb from rabbit and duck. In the ordinate are plotted the percentage of the total KFAb remaining in the circulation; the total KFAb was determined by the sum of the antibody bound in the kidney and the antibody circulating. The abscissa represents time in hours after injection of NTAb.

of 34 and 52 days. The preparation of duck γ_{2A}- and γ_{2B}-NTAb had long half-times in kidneys and adrenals (58.6 days in the kidneys and 59.0 days in adrenals). However, two different preparations of duck γ_{1M}-NTAb showed half-times in the kidney of 9.0 and 6.6 days.

V. Amounts of Available Renal Antigens.—Three different preparations of rabbit NTGG and two of duck NTGG were used in these studies. With rabbit NTGG the amount of antibody fixed in the kidneys was more or less proportional to the dose of KFAb injected with doses of 350 μg or less. However, with injections of 450 μg or more the amount fixed in the kidneys remained constant at 275 μg apparently reflecting a saturation of available renal antigen (Fig. 5). The kidneys of rats receiving 400 to 500 μg of KFAb were examined by the

fluorescent antibody echnique for localization of rabbit gamma globulin. It was localized exclusively to the glomerular capillary walls.

With the duck NTGG even at very high doses there is still increased fixation of antibody in the kidney. As seen in Fig. 5, the resulting curve does not flatten or plateau as in the case with the rabbit NTGG. It appears from this experi-

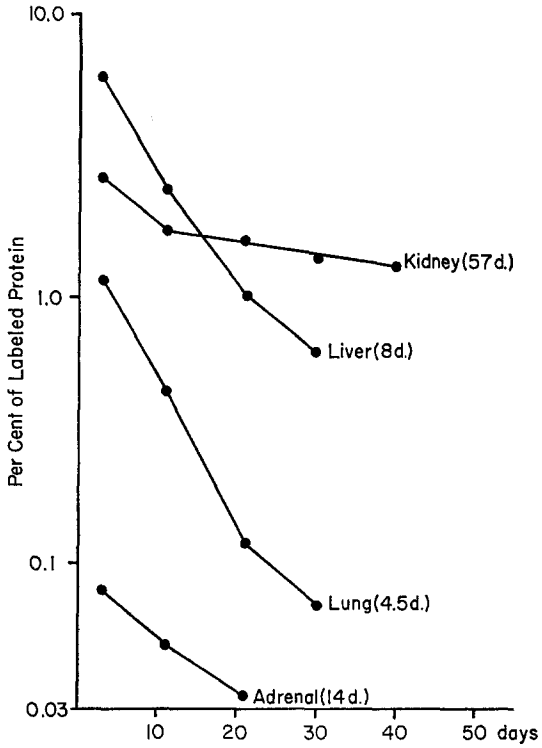

FIG. 4. Persistence in the tissue of NTAb from a representative rabbit antiserum. In the ordinate are plotted the percentage of labeled NTAb fixed in each tissue and the abscissa represents days after injection. Each point is the average of 3 to 5 rats. Figures in parentheses represent half-disappearance time in days.

ment that there is not complete saturation of antigenic sites of the kidney with duck NTAb in the doses employed. The kidneys of rats receiving 700 to 900 μg of KFAb were examined by fluorescent antibody technique for localization of duck gamma globulin and was found exclusively localized in the glomerular capillary walls.

DISCUSSION

Through the use of isotope-labeled NTAb it has been possible in these experiments to determine the pathologic effects of known numbers of NTAb mole-

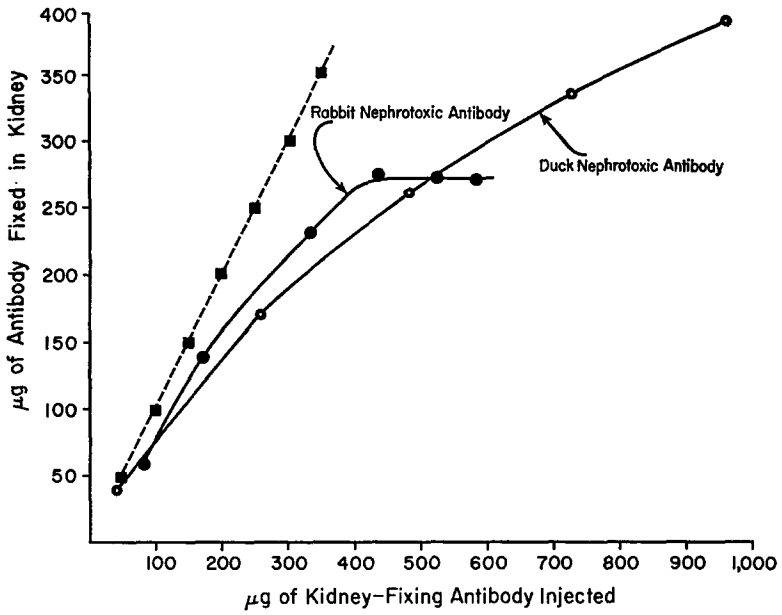

FIG. 5. Attempts to saturate available renal antigenic sites with rabbit and duck NTAb. The ordinate represents amounts of antibody fixed in the kidney and the abscissa represents the total amount of antibody injected. The interrupted line represents a theoretical curve in which all injected antibody would fix in the kidney.

cules reacting in the kidneys. As would be expected with both rabbit and duck NTAb there exists a direct relationship between amount of antibody injected, the amount fixing in the kidney, and the degree or severity of the immediate renal injury. As far as could be determined there was no qualitative difference among the light or the heavy NTAbs found within a given species. Comparable amounts of KFAb from any rabbit NTS produced comparable nephritis and the same was true for the various duck NTS. However, the amount of antibody needed to induce an immediate increase in glomerular permeability was greater than anticipated and apparently reflects the ability of the nephron to withstand the acute effects of antigen-antibody reactions. Approximately 175 μg of rabbit KFAb bound in the two kidneys of a rat is needed to produce a significant proteinuria; this represents 7.6×10^{14} molecules of gamma globulin.[1] The two kidneys of the rat contain approximately 60,000 glomeruli which means 1.2×10^{10} molecules of NTAb fixed per glomerulus. The two kidneys have a total filtration surface of about 14,000 mm^2 (17) or 2.3×10^{11} $m\mu^2$ per glomerulus. This means that one molecule of antibody is fixed per 20 $m\mu^2$ of

[1] Calculations based on a molecular weight of rabbit gamma globulin of 140,000 and Avogadro's number 6.02×10^{23}.

capillary filtration surface. Accepting dimensions of the gamma globulin molecule of 3 x 25 mμ and cross-sectional areas from approximately 9 to 75 mμ² this would mean that in order to produce immediate proteinuria 45 per cent or more of the filtration area must be physically covered by antibody molecules; the exact amount would depend upon the orientation of the antibody molecules to the capillary surface. The smaller cross-sectional area of a γ_2-globulin molecule is comparable to the estimated dimension of the antibody combining site, 8 to 9 mμ² (18, 19). This calculated extent of coverage of the capillary surface by 175 μg KFAb is in good agreement with the experimental observation that approximately 275 μg of rabbit KFAb completely saturates the available renal antigenic sites. 275 μg of antibody would physically occupy 71 per cent or more of the capillary surface depending again upon the orientation of the antibody molecules. These results indicate that the development of immediate detectable renal injury by a single injection of NTAb demands the involvement of at least half of the total filtration surface or the available antigenic sites of the glomerulus. Chronic continuing antigen-antibody reactions in the glomeruli as discussed in the following paper (20) can induce renal abnormalities with involvement of a much smaller portion of the glomerular surface.

Comparable calculations for duck NTAb are less certain since the sizes and shapes of these molecules are not well established. However, if the assumption is made that the γ_{2A}-(5.8S) and γ_{2B}-(7.4S) molecules have molecular weights comparable to that of rabbit γ_2-globulin and that the γ_{1M}-molecules of both species are of similar size some estimates may be made. The amount of duck γ_{2A}- and γ_{2B}-KFAb needed to give a minimal significant proteinuria is 150 μg which would be 0.9×10^{10} antibody molecules per glomerulus or 1 antibody molecule for every 26 mμ² of filtration surface, a value quite comparable to that for rabbit NTAb. However, the amount of duck γ_{1M}-KFAb needed to give immediate proteinuria was only 27 μg or 2.7×10^8 antibody molecules per glomeruli or 1 antibody molecule per 852 mμ² of filtration surface. This greater potency of the heavy antibody in inducing glomerular injury is similar to the greater hemolytic activity of 19S antibody (21).

The differences between the courses of proteinuria following the administration of duck and rabbit NTAb are probably related to the differences in mediation of the two immunological injuries. At levels of 175 to 200 μg of KFAb the rabbit NTAb induced a slight to moderate immediate proteinuria which extended beyond the initial 24-hour period; however, at these same levels duck NTAb induced a more severe immediate proteinuria that rapidly subsided. Only at levels of 250 μg or more of duck KFAb did proteinuria extend beyond the initial 24-hour period. Host complement which mediates at least in part, the injury produced by rabbit NTAb and is not significantly involved in the duck NTAb induced injury, may be related to the more persistent injury

induced by moderate doses of rabbit NTAb. It has been observed that complement fixes continually in the glomeruli during the 1st several weeks after injection of rabbit NTAb (12). However, it is clear that if severe enough injury to the glomerulus is produced by large amounts of duck NTAb without detectable participation of complement the resultant proteinuria will persist.

The NTAbs of different molecular types differed in physical and immunochemical properties and nephritogenic potency. The rabbit NTAb activity in the long-term immunized animals resided wholly in the γ_2-globulin in keeping with the paucity of 19S gamma globulin in this species. Duck KFAb and nephrotoxicity resided in three different immunoglobulins. Two of these (γ_{2A} and γ_{2B}) behaved electrophoretically identical with the mammalian γ_2-globulin but had sedimentation coefficients of 5.8 and 7.4S respectively; the third immunoglobulin was similar to the mammalian γ_{1M} both on electrophoresis and ultracentrifugation. Grey has previously shown antibody activity in γ_{2A} and γ_{2B} of ducks immunized to bovine albumin and has reported the susceptibility of the latter (his $\beta 2$ protein) to mercaptoethanol (22). Two differences were noted in the biological behaviour of the two preparations: (*a*) the preparation containing γ_{1M} was 10 times more nephritogenic on a weight basis than those containing γ_{2A} and γ_{2B}; and (*b*) the half-times of the two preparations in the kidney were markedly different (58.6 days for γ_{2A} and γ_{2B} and 7.3 days for γ_{1M}). The nephritogenic potency of γ_{2A}- and γ_{2B}-NTAb was only partially lost after treatment with mercaptoethanol, while that of γ_{1M}-NTAb was completely lost. However, neither the light γ_2-NTAbs nor the heavy γ_{1M}-NTAbs lost their ability to fix to the kidneys after treatment with mercaptoethanol. This is in agreement with reports which have shown the persistence of the ability of subunits of $\beta 2$ macroglobulin to fix to antigens after mercaptoethanol treatment (23, 24).

Finally the observations on the fixation and persistence in the circulation of NTAb gives some idea of the dynamics of the antigen-antibody reactions involved in NTN. Within 10 minutes after intravenous injection of NTAb 60 to 80 per cent of the antibody fixes in the kidney and maximum fixation, approximately 90 per cent of the total KFAb, occurs within 1 hour. However, 10 per cent of the KFAb remains in the circulation during the first 24 hours and probably for considerably longer. At the doses of NTAb employed there is no saturation of antigenic sites in the kidney which might account for the persistence of circulating antibody (6). Earlier findings by Pressman and associates (5, 6) and by Sarre and Wirtz (25) are in agreement with these observations. Characterization of the antibody in whole NTS and of the 10 per cent of the NTAb remaining in the circulation 1 hour after injection reveals significant differences. The antibody remaining in the circulation 1 hour postinjection fixed to the kidney more slowly than does the whole NTAb (40 per cent in 10 minutes, *vs.* 90 per cent). Also the persistence in the kidney of a major portion

of this circulating antibody was of shorter duration than that of the whole NTAb. The simultaneous presence of kidney fixed and circulating NTAb may be explained in several ways. Since the NTAb fixes initially to a variety of tissues other than the kidney from which they are lost relatively rapidly, some of the circulating NTAb may be antibody dissociated from extrarenal tissues. That this occurs is suggested by parabiotic experiments in which rats with well developed NTN when nephrectomized and united to normal partners show a small but continuous release of KFAb from extrarenal sites which fix in the kidneys of the normal parabiont (26). Another explanation is perhaps quantitatively more significant. In all likelihood any NTS contains KFAb of varying degrees of avidity. *In vivo* population of more avid molecules would rapidly fix to the kidney (and to a lesser extent to other tissues) and dissociate only slightly; the less avid antibody would fix and dissociate easily establishing an equilibrium between fixed and circulating antibody comparable to that observed in these experiments. The apparent continuous release and fixation of antibody in the glomeruli is consistent with previous observation of continuous fixation of complement in the glomeruli during the first several days after injection of NTAb. Such a continuing antigen–antibody-complement reaction could explain the progressive glomerular injury noted during this period. A comparison of the nephrotoxicity of the more avid and the less avid, easily dissociable NTAb's, is not possible from the present data. However, on the basis of observations made with hemolytic antibodies (27, 28) it could well be that the less avid antibodies might cause more injury in the glomeruli *via* repeated fixation and dissociation than the more avid antibodies.

CONCLUSION

A direct correlation between the amount of kidney-fixing antibody and the degree of associated renal injury was demonstrated for rabbit and duck nephrotoxic antibodies. No evidence for a qualitative difference among nephrotoxic antibodies of a given type was obtained. It appeared that duck nephrotoxic antibody was directed against a wider spectrum of rat renal antigens than was rabbit nephrotoxic antibody. In order to produce immediate proteinuria an amount of rabbit or duck gamma-2 kidney-fixing antibody capable of occupying approximately 45 per cent or more of the capillary filtration surface was needed. For immediate proteinuria an amount of rabbit gamma-2 kidney-fixing antibody capable of reacting with more than one-half of the available antigenic sites was needed. Less than twice that amount of rabbit antibody completely saturated available antigenic sites in the kidney. Virtually all nephrotoxic antibodies in hyperimmune rabbit nephrotoxic sera were of the gamma-2 variety while nephrotoxic antibodies in comparable duck nephrotoxic sera were found in gamma-2 (with 5.8 and 7.4S sedimentation coefficients) and gamma-1M fractions.

Gamma-1M duck nephrotoxic antibody was 60 times more potent a nephritogen than gamma-2 duck nephrotoxic antibody on a molecular basis. Mercaptoethanol abolished the nephrotoxicity of gamma-1M duck antibody and reduced that of gamma-2 duck antibodies but had no effect on rabbit gamma-2. In no case did mercaptoethanol treatment have an effect on the kidney-fixing properties of the antibodies involved.

After injection of nephrotoxic antibodies there appeared to be a prompt fixation of a majority of the antibody to tissue antigens primarily in the kidney. However, a small amount of potentially kidney-fixing antibody remained in the circulation for a considerable period apparently reflecting a dissociation of less avid antibodies with an equilibrium between fixed and free antibody. The role of this more easily dissociable antibody in the progression of nephrotoxic nephritis is not certain but it is possible that it could play a role in the early progression of the disease.

We are grateful to Dr. David D. Porter who performed the ultracentrifuge studies, to Dr. Howard Grey and Dr. Joseph D. Feldman for criticisms and advice, to Patricia McConahey for technical assistance.

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