EXPERIMENTAL ALLERGIC GLOMERULONEPHRITIS INDUCED IN THE RABBIT WITH HETEROLOGOUS RENAL ANTIGENS*,‡

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The immunization of experimental animals with heterologous or homologous renal antigens usually leads to the production of antikidney antibodies and, in some instances, to the development of glomerulonephritis. Early reports on the induction of glomerulonephritis by immunization of rats with renal antigens mixed with streptococcus (1) could not be confirmed (2, 3). However, in 1962, Steblay reported the development of a fulminant proliferative glomerulonephritis in sheep immunized to heterologous (4) and later homologous (5) glomerular basement membranes (GBM). The nephritis was transferable by serum (6, 7) and was apparently caused by an antibody which fixed to GBM (6). A similar nephritis has been reported also in monkeys (8) and goats (9) immunized to heterologous GBM, but immunopathologic studies are unavailable. Rabbits are known to develop antibodies reactive with homologous kidney following immunization with mouse (10), dog (11), rat (12), and rabbit (13) renal antigens. Immunization with homologous antigen has less frequently led to the development of nephritis (14).

A quite different kind of immunologically induced membranous nephritis was reported in 1959 by Heymann et al. (15) in rats immunized to homologous and later heterologous (16) non–GBM renal antigens mixed with complete Freund's adjuvant. The nephritis was not transferable by serum (17). The pathogenetic mechanisms in this nephritis have not been demonstrated completely but there is evidence to indicate that immune complexes unrelated to the glomerulus may play the most important pathogenetic role (18). A similar nephritis was also produced in rats by Blozis et al. (19) using similar antigens mixed with killed *Hemophilus pertussis*.

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The present report concerns the response of the rabbit to immunization with renal glomerular and tubular basement membrane antigens from several animal species differing widely in their phylogenetic relationship with the host. It shows that: (*a*) immunization with heterologous renal basement membranes leads to the production of antibodies, some of which fix to the host's kidneys; (*b*) these kidney-fixing antibodies (KFAb) react only with those antigens common between the immunizing kidney and the host kidney; (*c*) glomerulonephritis may develop if sufficient KFAb is formed, provided the host's kidneys have enough common antigen to allow the fixation of a nephritogenic amount of antibody; and (*d*) this type of nephritogenic response is not dependent on the use of Freund's adjuvant. A second report will consider the response of rabbits to homologous and autologous renal antigens (20).

Materials and Methods

Experimental Animals.—The immunizations were carried out in male Flemish giant and albino rabbits weighing 3–4 kg. The recipients for the studies of localizing antibodies were 2 kg albino rabbits and 130–150 g male Sprague Dawley rats.

Antigens.—Two renal antigenic preparations were used: (*a*) sediment—whole kidneys were homogenized in a Waring Blendor with 0.15 M saline and centrifuged at 2500 *g* for 30 min; the sediment was obtained, washed three times with saline, twice with distilled water, and lyophilized. This consisted essentially of basement membranes of glomeruli and tubules and collagenous elements of connective tissue. (*b*) GBM—prepared by the method of Krakower and Greenspon (21). Sediments from rat heart, striated muscle, and spleen were prepared in the same way as the renal sediment.

Fluorescent Antibody Methods.—Fluorescent antibody studies were made by conventional methods previously described (22). The following fluoresceinated antisera were used: (*a*) sheep anti–rabbit gamma globulin (GG)–rabbit GG was obtained by DEAE-cellulose column chromatography using 0.0175 M phosphate buffer at pH 6.5; (*b*) guinea pig anti–rabbit β_{1C}-globulin–β_{1c}-globulin was prepared by the zymosan method (23); the proportion of zymosan to rabbit sera was 1.35 mg per ml; (*c*) guinea pig anti–rabbit fibrinogen–fibrinogen was obtained by the method of Kekwick, et al. (24); (*d*) rabbit anti–rat GG and anti–rat β_{1C}-globulin, prepared as reported previously (22); (*e*) rabbit anti–guinea pig β_{1C}-globulin, obtained from Dr. C. G. Cochrane. All the antisera, after appropriate absorptions, reacted exclusively with their antigens as judged by immunoelectrophoresis. The GG fractions of the antisera were conjugated to fluorescein isothiocyanate by the method of Clark and Shepard (25). In vitro complement fixation test on the tissue sections was done as described by Klein and Burkholder (26), but using approximately one $C'H_{50}$ unit of guinea pig complement per slide and fluorescent anti–guinea pig β_{1C}-globulin.

Antikidney Antibodies.—The following methods were used to investigate antibodies which reacted with rabbit kidneys:

1. KFAb, i.e., those capable of fixing in vivo to the kidneys, were measured by a method similar to the one described by Pressman and Keighley (27) and detailed previously (28). GG or globulin from sera of immunized rabbit were obtained by DEAE-cellulose chromatography at 0.0175 M, pH 6.5, or by ammonium sulphate precipitation at 50% concentration, respectively. They were labeled with ^{131}I (29) and injected intravenously into two or three normal rabbits in doses of 200–400 μg of protein containing 5–20 μc of ^{131}I. The rabbits were sacrificed 1–3 days after injection, the kidneys were perfused *in situ* with saline, removed, and

assayed for radioactivity in a NaI crystal scintillation counter. Serum was also counted in order to establish correction figures for the presence of nonantibody GG radioactivity in the kidneys. The latter figures were derived from control experiments in which rabbits were injected with ^{131}I-labeled normal rabbit GG and treated in the same way. In most instances, the kidneys were then homogenized and fractionated into sediment and supernatant and the radioactivity of each determined. With selected antisera the liver and lung were also counted. The figures are expressed either in relative terms as percentage of injected dose fixed in the organ, or in absolute terms as μg of KFAb.

In the group of rabbits immunized with rat kidney sediment, a second approach was used to investigate KFAb. ^{131}I-labeled GG from the rabbit sera was absorbed heavily with rabbit kidney sediment (1.0–1.2 mg of GG per 20 mg of sediment). The absorbed and unabsorbed GG's were injected into rats which were sacrificed 3 days later and the KFAb determined. The difference in fixation between the two preparations was taken as evidence of cross-reacting antibodies to rabbit kidney.

2. Antibodies capable of fixing in vitro to recently sonicated renal sediment or GBM were investigated using ^{131}I GG from immunized rabbits. Usually 10–20 μg of GG was mixed with 100–1000 μg of sediment or GBM. The mixtures were incubated at 37°C for 1 hr and then left in the cold overnight; they were then centrifuged, resuspended, and washed twice with cold saline and the precipitates counted. Control studies were done using ^{131}I normal rabbit GG.

3. Precipitating antibodies to a variety of renal antigens were investigated by the micro-Ouchterlony method. The antigens were recently sonicated GBM, renal sediment, or renal extract. The extract was the supernatant of the kidney sediment preparation after the first centrifugation.

4. Rabbit antibodies capable of fixing to kidney frozen sections were investigated by the indirect (double layer or sandwich) fluorescent antibody method (30). The sera, undiluted and diluted severalfold, were applied to normal kidney sections; after incubation for 45 min, the slides were washed and stained with fluorescent sheep anti-rabbit GG.

Miscellaneous.—The immunized rabbits were periodically examined for proteinuria by analyzing 24-hr collections of urine. Proteinuria was determined by the sulfosalicylic acid method (31) and expressed in mg per 24 hr. Normal rabbits have, at the most, traces of protein in the urine with total urinary volumes usually varying from 50–200 ml per day.

Anti-GG factors were determined by agglutination of sheep erythrocytes coated with rabbit hemolysin (32).

Immunoconglutinin was determined as described by Lachmann and Liske (33) using rabbit sera as the source of complement.

EXPERIMENTAL

Immunopathology of Rabbits Immunized with Renal and Nonrenal Antigens

Groups of rabbits were immunized with renal sediment or GBM from one of five different species. The details of the immunizations are given in Table I. Note that in groups 1 and 3 the sediment was administered intraperitoneally without adjuvant, as has been done to obtain a potent heterologous nephrotoxic serum (22). No effort was made to vary the amounts of antigen. Rabbits were examined for proteinuria every 5–7 days. Those developing proteinuria were usually checked more frequently and were subjected to renal biopsy at appropriate times. Rabbits were sacrificed from 100–165 days after immunization, at which time tissues were obtained for pathological and immunohistochemical studies.

In the groups of rabbits immunized to rat kidney sediment in saline (group 1, Table I), an attempt was made to correlate the titer of antibodies to the rat kidney and the appearance of

proteinuria in the immunized rabbits. The amounts of antibody in the various rabbit antisera were judged by the amounts of immediate proteinuria they would induce when injected into rats (28). The rabbits were bled 16 days after the last immunization. The sera, after decomplementation by heating at 56°C for 1 hr and absorption with an excess of rat erythrocytes, were injected in 1.0 ml amounts into rats. Proteinuria was determined 24 and 72 hr later.

TABLE I

Antigens and Immunization Schedules

Antigen	Route	Schedule	Total Amount Administered
			mg
Renal:			
1. Rat sediment in saline	Intraperitoneal	Three consecutive daily injections; rest 4 days and repeat. Rest 8–12 wks and repeat 6 injections	First course: 220 Second course: 180
2. Rat sediment in CFA*	Foot-pads and subcutaneous	Three weekly injections‡ followed by 4–6 bi-weekly injections	75–100
3. Human sediment in saline	Intraperitoneal	As Group 1	First course: 220 Second course: 180
4. Human sediment in CFA	Foot-pads and subcutaneous	As Group 2	75–100
5. Human GBM in CFA	Foot-pads and subcutaneous	As Group 2	90
6. Duck (Pekin, *Psophia virdis*) sediment in CFA	Foot-pads and subcutaneous	As Group 2	90
7. Turtle (*Chrysemys picta*) sediment in CFA	Foot-pads and subcutaneous	As Group 2	90
8. Frog (*Leptodactylus pentodactylus*) sediment in CFA	Foot-pads and subcutaneous	As Group 2	45
Nonrenal:			
9. Rat spleen sediment in CFA	Foot-pads and subcutaneous	Three weekly injections; rest 8–10 wk. Repeat 4 weekly injections	270
10. Rat heart sediment in CFA	Foot-pads and subcutaneous	" "	210
11. Rat muscle sediment in CFA	Foot-pads and subcutaneous	" "	270

* Freund's adjuvant contained 1 mg of *Mycobacterium tuberculosis* H37Ra in 1.0 ml.

‡ Antigen administered in 1.0 ml volumes.

Experiments were done in order to determine the specificity of the circulating rabbit antibodies which reacted with homologous kidney sediment. 300 μg of ^{131}I-labeled GG from rabbits 93–50 and 92–13 immunized to rat and human kidney sediments, respectively, (Table III), were reacted in vitro with 100 μg of rabbit GBM as described before. The same preparations were absorbed with an excess of the heterologous sediment used for immunization (300 μg of GG was mixed with 5 mg of sediment at 37°C for 1 hr and in the cold for 16 hr) and then tested for in vitro fixation to rabbit GBM.

Experiments were carried out in order to determine the specificity of the glomerular bound GG in the immunized rabbits. Kidneys from nephritic rabbits 93–50 and 92–13 immunized to rat and human renal sediment, respectively, were obtained at sacrifice. These kidneys were

known to contain glomerular fixed GG in previous biopsy specimens. The kidneys were fractionated into 2500 *g* sediment and supernatant; the sediment was washed five times with saline and then mixed for 30 min with 0.1 M sodium citrate, pH 2.5; the mixture was centrifuged at 2500 *g* for 30 min; the supernatant was obtained, neutralized to pH 7.0, and concentrated by pressure dialysis. The presence of antibody to rabbit kidney in the eluates was looked for by the indirect fluorescent antibody method.

Finally, for the purpose of determining the organ specificity of the antikidney response, other groups of rabbits were immunized to sediments from rat muscle, heart and spleen in complete Freund's adjuvant (Table I). These rabbits also were used for other experimental purposes and their schedule of immunization (Table I) differed from the schedule of the groups immunized with renal antigens. They had, at the most, three determinations of protein in their urine. Immunohistochemical and pathologic studies of the kidneys were made on surgical biopsies obtained 30 and 120 days after the first immunization.

Immunology of Cross-Reacting Renal Antigens

As the results of the above experiments suggested that rabbits immunized to heterologous kidneys made antibodies to antigenic components common between their kidneys and the immunizing ones, experiments were done to determine the extent of common antigen(s) among kidneys of different species.^{131}I-labeled GG from pooled rabbit antisera to human, duck, and frog kidney sediments (groups 4, 6, and 8, respectively, Table I) were injected in increasing doses from 0.2–65 mg into *rats*. The rats were observed for proteinuria and sacrificed 3 days later. Their kidneys, livers, lungs, and spleens were counted for radioactivity and studied immunohistochemically and histologically. This approach (28) afforded measurements of: (*a*) the quantity of rat KFAb in the various rabbit antisera; (*b*) the quantity of antigen in the rat kidneys capable of reacting with the rabbit antibodies to heterologous antigens; and (*c*) the resultant degree of renal injury. In a similar fashion, globulin fractions from pools of guinea pig antisera to human and duck kidneys were studied for their ability to fix to rabbit kidneys. The antisera were obtained from guinea pigs immunized with a total of 30 mg of renal sediment in complete Freund's adjuvant over a period of 3 wk.

Another experiment was carried out to determine if a second or autologous phase of nephrotoxic serum nephritis (34) could develop in the rats injected with saturating, but nonnephritogenic, amounts of rabbit anti-duck kidney GG. Two rats were injected intravenously with rabbit anti-duck kidney GG containing the saturating dose of KFAb and were simultaneously immunized to normal rabbit GG by subcutaneous injection of 200 μg in Freund's incomplete adjuvant. They were checked weekly for proteinuria. When proteinuria developed, the rats were sacrificed and the kidneys were obtained for pathological and immunohistochemical studies.

RESULTS

Immunopathology of Rabbits Immunized to Renal and Nonrenal Antigens

Clinical Studies.—A summary of all results is given in Table II and detailed data on the rabbits that became proteinuric are listed in Table III. Note that among the rabbits with transitory proteinuria, there was no relationship between the degree and duration of proteinurias. Thus, rabbit 82-64 (Table III) had the most severe proteinuria, 1065 mg, but it lasted only 3 days, while rabbit 82-63 had a mild proteinuria which lasted for 17 days. Rabbit 93-50, which developed a persistent and progressive disease, had proteinuria ranging around

700 mg during the first 3 months, increasing progressively to levels of 1300–1600 mg during the final 2 months before sacrifice.

Immunization with small amounts of rat kidney sediment in Freund's adjuvant was less nephritogenic than immunization with four times greater amounts of rat kidney sediment in saline (groups 1 and 2, Table II). The nephritogenicity of antigens from different species varied. Immunization of 11 rabbits with rat and human kidney sediment and human GBM in Freund's adjuvant

TABLE II

Proteinuria and Immunohistochemistry

Immunization	Proteinuria/No. rabbits	Fluorescent antibody* (No. positive/No. studied)	
		GG	β1C-globulin
Renal antigens:			
1. Rat sediment in saline	4/9	4/4	4/4
2. Rat sediment in CFA‡	1/4	3/4	3/4
3. Human sediment in saline	0/4	4/4	—
4. Human sediment in CFA	1/3	3/3	2/3
5. Human GBM in CFA	1/4	3/4	1/3
6. Duck sediment in CFA	0/10	5/10	0/10
7. Turtle sediment in CFA	0/4	2/4	2/4
8. Frog sediment in CFA	0/4	2/4	0/4
Nonrenal antigens:			
9. Rat spleen sediment in CFA	1/3	1/3	1/3
10. Rat heart sediment in CFA	0/3	0/3	0/3
11. Rat muscle sediment in CFA	0/4	0/4	0/4

* Refers only to glomerular capillary walls.

‡ Complete Freund's adjuvant.

(groups 2, 4, and 5) induced proteinuria in three instances while immunization with kidney sediments from duck, turtle, and frog in adjuvant (groups 6, 7, and 8) failed to induce proteinuria in any of 18 rabbits.

Rabbits immunized to sediments from rat muscle and heart did not develop proteinuria. One of three rabbits immunized to rat spleen sediment had mild proteinuria in urine samples obtained 120 days after the first immunization and 60 days after the last.

Immunohistochemical and Histologic Studies (Table II).—A majority of rabbits immunized with renal antigens had fixation of GG to their glomerular capillary walls detectable by fluorescent antibody 60 to 140 days after the first immunization (Fig. 1). GG was distributed in a uniform, linear pattern identical to the distribution of anti-GBM antibody in nephrotoxic serum nephritis. In the rabbits immunized to rat kidney sediment in saline, GG was present in glomeruli

as early as 12 days after immunization. The incidence of rabbits showing glomerular fixed GG was higher in the groups immunized to kidneys of mammalian species (17 of 19) than in the groups immunized to kidneys of non-mammalian species (9 of 18). All rabbits developing proteinuria had, besides fixation of GG, fixation of β_{1C}-globulin to the glomeruli; however, only 30% of those without a proteinuria but with fixed GG had β_{1C}-globulin fixed specifically to the glomeruli.

In two of the rabbits showing transitory proteinuria (82-64 and 21-63, Table III), the intensity of the reactions for GG and β_{1C}-globulin fluctuated. Strong

TABLE III

Description of Glomerulonephritis in Rabbits

Rabbit No.	Renal antigen	Proteinuria			Glomerular-fixed GG	Circulating KFAb	Glomerular lesion
		Started: (day after 1st immunization)	Lasted:	Highest figure			
			days	*mg/24 hr*			
93-50	Rat sediment in saline	10	165*	1613	+++	+	Severe; membranous
93-51	Rat sediment in saline	13	6	296	++	+	Mild; membranous
82-63	Rat sediment in saline	10	17	54	+	−	Mild; membranous
82-64	Rat sediment in saline	10	3	1065	++‡	−	Mild; membranous
85-69	Rat sediment in CFA§	50	7	402	+	Not done	Mild; membranous
92-13	Human sediment in CFA	125	9*	792	+++	−	Severe; membranous and proliferative
21-63	Human GBM in CFA	70	7	210	++‡	Not done	Mild; membranous
10-87	Rat spleen sediment in CFA	120	14	7	++	Not done	Mild; membranous

* Present until sacrifice.

‡ Concentration of GG decreased after proteinuria subsided.

§ Complete Freund's adjuvant.

to moderate reactions were seen in biopsies taken at the time of proteinuria and weak or negative reactions were seen in biopsies taken 8–10 wk later. This variation in the amounts of glomerular fixed GG was not observed in rabbits 93-51 and 82-63, which also had transitory proteinuria. Rabbit 85-69 could not be evaluated in this respect for he was biopsied only once.

The rabbit immunized to rat spleen, (10-87), which had proteinuria, had linear fixation of GG and β_{1C}-globulin to the glomerular capillary wall.

Fibrinogen (and/or fibrin) was observed in an irregular distribution along glomerular capillary walls only in rabbit 92-13 which had a severe nephritis.

Histopathologically, the rabbits exhibiting transitory proteinuria showed mild glomerular changes consisting of focal basement membrane thickening best seen at the time of proteinuria. Rabbit 93-50 (Table III) which had persistent

proteinuria had mild focal glomerular changes when biopsied 35 days after the initial immunization; however, the pathologic changes progressed to a severe, diffuse membranous glomerulonephritis with scarring and synechiae. Rabbit 92-13, which became severely nephritic after immunization with human kidney, had extensively damaged glomeruli: thickened basement membranes, polymorphonuclear leukocyte (PMN) infiltration, adhesions, synechiae, and lobulation.

Immunologic Studies.—Circulating KFAb capable of reacting with rabbit kidney was investigated in selected rabbits. Two of five rabbits (93-50 and 93-51, Table III) immunized to rat kidney sediment in saline had KFAb. One of them (93-50) which developed a persistent proteinuria, had low levels on two occasions, 35 and 160 days after immunization (0.02 and 0.08% fixation to rabbit kidneys, respectively). The other (93-51), which developed an episode of transitory proteinuria, had KFAb only at the time of proteinuria and shortly thereafter. The KFAb in this rabbit was only detectable by injection into rats after prior absorption with rabbit kidney sediment. Of the three rabbits with no circulating KFAb on three different occasions, two had transitory proteinuria (82-63 and 82-64, Table III). KFAb was absent from the serum of rabbit 92-13 immunized to human kidney sediment and developing severe nephritis. However, the same serum had a small degree of in vitro fixation to rabbit GBM. A pool of sera from the groups of nonnephritic rabbits immunized to human and frog sediment in Freund's adjuvant (groups 4 and 8, Table I) did not contain KFAb for rabbit's kidney. Precipitating antibodies to rabbit sediment or GBM could not be demonstrated in gel in any of the rabbits.

There was no correlation between the titer of antibodies to the rat kidney and the appearance of proteinuria in the rabbits immunized to rat kidney sediment in saline. Five of nine rabbits' sera, injected in 1.0 ml amounts, induced an acute nephritis in rats. Only two of these came from rabbits developing proteinuria and three were from rabbits without proteinuria. The remaining four rabbit sera did not induce proteinuria in rats and two came from proteinuric rabbits.

^{131}I-labeled GG from rabbits 93-50 and 92-13 immunized to rat and human kidney sediment, respectively, fixed in vitro to rabbit GBM (6.0 and 2.0% fixation as compared to 0.8% for normal GG). This fixation was blocked if these preparations were previously absorbed with the kidney sediment used for immunization (1.0 and 0.8%, respectively), suggesting that the antibody was directed only to the antigenic determinants in the rabbit GBM, which were shared or cross-reacting with the immunizing kidney.

Eluates from kidneys of rabbits 93-50 and 92-13 immunized to rat and human kidney, respectively, fixed specifically to normal rabbit glomeruli, as demonstrated by the indirect fluorescent antibody method; furthermore, the eluate of rabbit 92-13 also fixed to tubular basement membrane. Eluates from two normal rabbit kidneys showed no kidney fixation.

Four of six rabbits immunized to rat kidney sediment in saline had anti-GG factors in their sera when tested 16 days after the last administration of antigen (three were proteinuric, 82-63, 93-50, and 82-64, Table III). Only one of them (82-63, Table III) had immunoconglutinin. All rabbits immunized with human kidney sediment in Freund's adjuvant had anti-GG factors when tested at day 140.

Immunology of Cross-Reacting Renal Antigens

By injection of large amounts of GG, it was possible to demonstrate that the antigens in a rat kidney would fix up to 250 μg of rabbit anti-human kidney GG. Previous studies in the rat showed that fixation of more than 150 μg of KFAb will induce an acute proteinuria (28). Rats fixing these amounts of rabbit anti-human kidney GG developed a mild, acute glomerulonephritis, as judged by proteinuria and histopathologic changes. However, the nephritis was much less severe than when comparable amounts of a rabbit anti-rat kidney GG were used. By fluorescent antibody, the injected rabbit GG was fixed only in the glomerular capillary walls. By isotope methods, the antibody was not organ specific. There was also localization of the antibody to the liver, lung, and spleen of the rat. The per cent of injected dose per g of organ was 0.27, 0.05, 0.22, and 0.31 in kidney, liver, lung, and spleen, respectively.

A rat's kidney would fix no more than 5 μg of rabbit anti-duck kidney GG, regardless of the amount of GG injected. Rats injected with this saturating amount did not develop immediate nephritis. However, a mild, autologous phase nephritis developed in rats previously immunized to rabbit GG 40 days after injection of the anti-duck KFAb. The nephritic rats showed linear fixation of rat GG and β_{1C}-globulin to the glomerular capillaries. This experiment demonstrates that the rabbit anti-duck kidney GG, after fixation in the rats' kidneys, was anatomically so situated that its further participation in antigen-antibody reactions caused a glomerulonephritis. Anti-duck kidney GG also had some localization in the rat's liver, lung, and spleen. Rabbit anti-frog kidney GG also fixed to the rat to a similar extent as the anti-duck kidney GG. However, anti-frog kidney GG did not fix to organs of the rat other than the kidney.

Guinea pig anti-human kidney cross-reacted extensively with rabbit kidney. In one experiment, up to 144 μg, which was not saturating, was able to fix to one rabbit kidney weighing 8 g. All of the antibody was localized in the kidney sediment, and by fluorescent antibody it was exclusively fixed to the glomeruli. Guinea pig anti-duck kidney GG did not fix to the rabbit's kidney. However, guinea pigs immunized to both these antigens exhibited fixation of their own GG to their glomeruli and some immunized with human kidney became nephritic.

DISCUSSION

Rabbits responded strongly to heterologous renal sediments with the production of antibodies and, in the majority, a small proportion of these antibodies

cross-reacted with their own glomeruli. If this small proportion of antibodies capable of reacting with the host's tissues may be regarded as autoantibodies, then the associated nephritis developing in some rabbits may be considered an example of autoimmunity, realizing that the initiating antigen is not an autoantigen. It is apparent that the state of natural immune tolerance to GBM, a tissue structure which is normally exposed to the circulation via its fenestrated endothelial covering, can usually be terminated by appropriate immunization. Rabbits not only responded to immunization with heterologous basement membrane but also to homologous and autologous basement membranes (20), albeit with inconsistent and usually transitory formation of autoantikidney antibody. However, the development of glomerulonephritis did not always occur with the appearance of such autoantibodies, as will be discussed later.

The autoantibodies were rarely demonstrable in the circulation by the in vivo technique of KFAb or by their in vitro fixation to GBM, but were regularly seen as GG in the host's glomeruli by the fluorescent antibody method. The antibodies were rarely found circulating, presumably because most of them were removed by the host's kidneys, making measurements of the amount synthesized impossible. A similar absence of antikidney antibodies in the circulation of intact sheep with autoimmune nephritis has been observed (6). In these sheep, circulating antikidney antibody appears after nephrectomy of the subject. That the GG in the glomeruli was antiglomerular autoantibody was supported by two observations: (*a*) material eluted from nephritic kidneys was shown to react with normal homologous glomeruli, and (*b*) the linear pattern of fixation of GG was that shown to be characteristic for anti GBM antibodies fixed to the glomerular capillary wall (18).

The autoantibodies, by in vitro fixation to GBM, were shown to be directed to those antigens common or cross-reacting between the immunizing kidney and that of the host. Common glomerular antigens have been demonstrated among several mammalian species. For example, nephrotoxic serum nephritis has been induced in dogs (35), mice (36), and rabbits (35, 37) by injection of antibodies directed to kidneys of other mammalian species. Gery et al. (38) measuring in vivo reactions of KFAb clearly showed the cross species reactivity of the antikidney antibodies among several mammals. Our experiments using rabbit antikidney sera in rats demonstrated a large amount of common antigen(s) shared between mammalian species and also showed the presence of smaller amounts of common glomerular antigen(s) shared between rats and species as phylogenetically distant as ducks and frogs. In the latter species, the kidneys do not develop beyond the mesonephric stage. The antigen(s) common between the nonmammalian species and rats probably doesn't exceed 3% of the available glomerular antigenic sites (as measured with rabbit antibody). It should be noted that heterologous sediments from muscle and heart, which share approximately 40% or more of available antigenic sites with the kidney

(as measured in the rat) (39) did not elicit detectable antikidney autoantibodies. Since kidneys of nonmammalian species, sharing only few sites, were effective, it would appear that the renal *specific* antigens were the most immunogenic in this regard. Still, the autoimmune antikidney response is not entirely specific for renal antigens since immunization with rat spleen induced autoantikidney antibodies and even nephritis on one occasion. Also, Rudofsky and Steblay have reported nephritis in sheep immunized with human lung (40).

An important consideration in the development of glomerulonephritis was the extent and duration of the autoimmune response of the rabbit. This was probably of most significance in those rabbits immunized to kidneys of mammalian species in which, as will be discussed later, the number of common available glomerular antigens in the host was not a limiting factor. In these rabbits, if the immune response to the common antigen(s) would have been large and continuous, one would have expected a high incidence of glomerulonephritis instead of the 25–44% incidence which was observed. Though, as discussed earlier, the amount of autoantikidney synthesized cannot be measured, several observations indicated that the autoimmune response of the rabbits was small and transitory. Thus, many rabbits had a self limited nephritis, presumably as a consequence of a temporary production of autoantibodies as indicated by: (*a*) the short lived presence of detectable, circulating KFAb, and (*b*) the decrease or disappearance of glomerular fixed GG after a few weeks. That the amount of antibody was small in the nonnephritic rabbits was implied from the absence of circulating KFAb, the poor fixation of GG in the glomeruli and the absence of β_{1C}-globulin in the glomeruli.

Finally, the development of glomerulonephritis also appeared to depend upon the amount of common antigen(s) in the host's glomeruli capable of fixing antibody formed to the heterologous kidney. From quantitative studies in nephrotoxic serum nephritis, it is known that the development of immediate or late renal injury depends upon the amounts of glomerular antigenic sites reacting with antibody (28, 34). If the number of antigenic sites was large and there was sufficient antibody to fix to them, nephritis would develop within a matter of hours (28). If, on the other hand, the number of antigenic sites was small, nephritis, if it developed at all, did so only after a prolonged period of antigen-antibody interaction (34). In this situation, the number of antigenic sites was the limiting factor in the development of renal injury. These data, if extended to the present experiments, may, in part, explain the difference in incidence of glomerulonephritis associated with the different immunizations. Though kidneys of distantly related species were immunogenic and did elicit autoantibodies, the small quantity of common antigen in the host kidney most probably would not have permitted the fixation of nephritogenic amounts of antibody, even if such were circulating. Thus, glomerulonephritis did not develop in any of the rabbits immunized with duck, turtle, and frog kidney sediment. On the

other hand, if immunization with kidneys of mammalian species causes production of autoantibody, the host kidney has ample antigenic sites with which this autoantibody can react and cause glomerulonephritis. In this situation, the amount of antikidney autoantibody is the limiting factor.

CONCLUSIONS

Rabbits immunized to different heterologous renal antigens developed antibodies some of which are fixed to their own glomeruli (autoantibodies). These autoantibodies, reacting with the host's kidneys, were directed to those antigenic determinants which were present in identical or cross-reactive form in both the immunizing antigen and the kidneys of the host. Glomerulonephritis developed in some of the rabbits immunized to heterologous mammalian kidneys, but in none of those immunized to nonmammalian kidneys. The development of glomerulonephritis in the immunized rabbits appears to depend upon two factors: (*a*) the amount of autoantikidney antibody made to the common renal antigens; and (*b*) the quantity of the common antigens available in the host's kidneys. An amount of common antigen sufficient to fix a nephritogenic amount of antibody is essential for the development of disease.

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BIBLIOGRAPHY

1. Cavelti, P. A., and E. S. Cavelti. 1945. Studies on the pathogenesis of glomerulonephritis: II. Production of glomerulonephritis in rats by means of autoantibodies to kidney. *Arch. Pathol.* **40**:158.
2. Humphrey, J. H. 1948. Pathogenesis of glomerulonephritis: Reinvestigation of autoimmune hypothesis. *J. Pathol. Bacteriol.* **60**:211.
3. Middleton, E. Jr., E. B. Middleton, and B. C. Seegal. 1953. Effect of injecting rats with homologous renal tissue mixed with adjuvants or streptococci. *Arch. Pathol.* **56**:125.
4. Steblay, R. W. 1962. Glomerulonephritis induced in sheep by injections of heterologous glomerular basement membrane and Freund's complete adjuvant. *J. Exp. Med.* **116**:253.
5. Steblay, R. W. 1965. Glomerulonephritis induced in sheep by injections of homologous sheep glomerular basement membrane (SGBM) and Freund's adjuvant. *Fed. Proc.* **24**:693.
6. Lerner, R. W., and F. J. Dixon, 1966. Transfer of ovine experimental allergic glomerulonephritis (EAG) with serum. *J. Exp. Med.* **124**:431.
7. Rudofsky, V., and R. W. Steblay. 1966. Studies on autoimmune nephritis in sheep. II. Passive transfer of nephritis in sheep by plasma. *Federation Proc.* **25**:569.
8. Steblay, R. W. 1963. Glomerulonephritis induced in monkeys by injections of heterologous glomerular basement membrane and Freund's adjuvant. *Nature.* **197**:1173.
9. Williams, R., and R. W. Steblay. 1965. Glomerulonephritis induced in goats by

injection of human glomerular basement membrane (HGBM) and Freund's adjuvant (FA). *Federation Proc.* **24**:243.

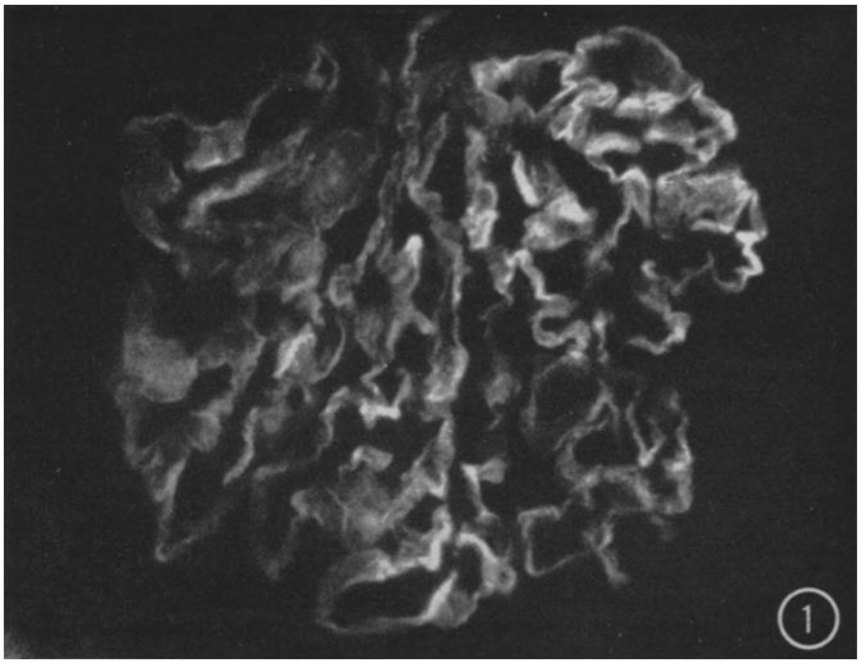
10. Furth, J., and E. A. Kabat. 1941. Immunological specificity of material sedimentable at high speed present in normal and tumor tissue. *J. Exp. Med.* **74**:247.
11. Simonsen, M. 1953. Studies on the pathogenesis of experimental glomerulonephritis. *Acta Pathol. Microbiol. Scand.* **32**:85.
12. Asherson, G. L., and D. C. Dumonde. 1963. Auto-antibodies production in rabbits. II. Organ specific auto-antibodies in rabbits injected with rat tissues. *J. Immunol.* **6**:19.
13. Schwentker, F. F., and F. C. Comploier. 1939. Production of kidney antibodies by injection of homologous kidney plus bacterial toxins. *J. Exp. Med.* **70**:223.
14. Milgrom, F., E. Centeno, S. Schulman, and E. Witebsky. 1964. Auto-antibodies resulting from immunization with kidney. *Proc. Soc. Exp. Biol. Med.* **116**:1009.
15. Heymann, W., D. B. Hackel, S. Harwood, S. G. Wilson, and J. L. P. Hunter. 1959. Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspension. *Proc. Soc. Exp. Biol. Med.* **100**:660.
16. Heymann, W., E. P. Kmetec, S. G. F. Wilson, J. L. P. Hunter, D. B. Hackel, and F. Cuppage. 1963. Experimental autoimmune renal disease in rats. *In* "Immunopathology", Third International Symposium. P. Grabar and P. Miescher, editors. Benno Schwabe & Co., Basel. 241.
17. Hess, E. V., C. T. Ashworth, and M. Ziff. 1965. Nephrosis in the rat induced by rat kidney extracts. *Ann. N. Y. Acad. Sci.* **124**:323.
18. Dixon, F. J., E. R. Unanue, and J. I. Watson. 1965. Immunopathology of the kidney. *In* "Immunopathology", Fourth International Symposium. P. Grabar and P. Miescher, editors. Benno Schwabe & Co., Basel. 363.
19. Blozis, G. D., B. Spargo, and D. A. Rowley. 1962. Glomerular basement membrane changes with the nephrotic syndrome produced in the rat by homologous kidney and hemophilus pertussis vaccine. *Am. J. Pathol.* **40**:153.
20. Unanue, E. R., F. J. Dixon, and J. D. Feldman. 1967. Experimental allergic glomerulonephritis induced in the rabbit with homologous renal antigens. *J. Exp. Med.* **125**:163.
21. Krakower, C. A., and S. A. Greenspon. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* **51**:629.
22. Unanue, E. R., and F. J. Dixon. 1964. Experimental glomerulonephritis. IV. Participation of complement in nephrotoxic nephritis. *J. Exp. Med.* **119**:965.
23. Mardiney, M. R., and H. J. Müller-Eberhard. 1965. Mouse β_{1C}-globulin: Production of antiserum and characterization in the complement reaction. *J. Immunol.* **94**:877.
24. Kekwick, R. A., M. E. MacKay, M. H. Nance, and B. R. Record. 1955. The purification of human fibrinogen. *Biochem. J.* **60**:671.
25. Clark, H. F., and C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. *Virology.* **20**:642.
26. Klein, P., and P. Burkholder. 1959. Ein Verfahren zur Fluoreszenoptischen Darstellung der Komplement-bindung und seine Anwendung zur Histoimmunologischen Untersuchung der experimentellen Nierenanaphylaxie, *Deut. Med. Wochschr.*, **84**:2001.
27. Pressman, D., and G. Keighley. 1948. The zone of activity of antibodies as

determined by the use of radioactive tracers; the zone of activity of nephrotoxic antikidney serum. *J. Immunol.* **51**:141.

28. 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.
29. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for in vivo studies. *Intern. Arch. Allergy Appl. Immunol.* **29**:185.
30. Coons, A. H., E. H. Leduc, and J. M. Connolly. 1955. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exp. Med.* **102**:49.
31. Kingsbury, F. B., C. C. Clark, G. Williams, and A. L. Post. 1926. Rapid determination of albumin in urine. *J. Lab. Clin. Med.* **11**:981.
32. Milgrom, F. 1962. Rabbit sera with "anti-antibody". *Vox. Sanguinis.* **7**:545.
33. Lachmann, P. J., and R. Liske. 1966. The preparation and properties of alexinated intermediates that react with conglutinin. II. *Immunology.* **11**:255.
34. Unanue, E. R., and F. J. Dixon. 1965. Experimental glomerulonephritis. VI. The autologous phase of nephrotoxic serum nephritis. *J. Exp. Med.* **121**:715.
35. Markowitz, A. S. 1960. Interactions of anti-glomerular basement membrane antisera. *Immunology.* **3**:117.
36. Arana, J., B. Nidus, J. Blair, and M. H. Kaplan. 1964. Nephrotoxic nephritis in the mouse. Evidence of species cross-reactive nephrotoxic antigen(s). *Federation Proc.* **23**:509.
37. Steblay, R. W. 1964. Immediate or delayed nephritis in rabbits induced by intravenous injection of sheep anti-human glomerular basement membrane sera. *Federation Proc.* **23**:216.
38. Gery, I., Y. Yagi, and D. Pressman. 1965. Reactions of localizing anti-kidney antibodies with tissues of various species. *J. Immunol.* **94**:950.
39. Katz, D. H., E. R. Unanue, and F. J. Dixon. 1967. Nephritogenic properties of cross-reacting kidney fixing antibodies to heart, spleen, and muscle. *J. Immunol.* **98**: In press.
40. Rudofsky, U., and R. W. Steblay. 1965. Glomerulonephritis induced in sheep by injections of human lung (HL) and Freund's adjuvant. *Federation Proc.* **24**:1963.

EXPLANATION OF PLATE 14

FIG. 1. Fluorescence micrograph of a glomerulus from rabbit 92–13 (Table III) made nephritic by immunization with human kidney sediment. Section is stained with fluorescent anti–rabbit GG. Rabbit GG is distributed linearly along the glomerular capillary walls. × 450.



(Unanue and Dixon: Glomerulonephritis and heterologous renal antigens)