Studies of the Antigens Involved in an Immunologic Renal Tubular Lesion in Rabbits

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Rabbits injected with nonglomerular components of rabbit kidney incorporated in Freund's complete adjuvant develop a lesion characterized by a) extensive interstitial fibrosis, tubular degenerative changes, and sparse focal lymphocytic infiltrates; b) the deposition of IgG and C3 in a granular pattern along the basement membranes of proximal convoluted tubules; and c) functional tubular defects if the lesions are severe. The antibodies were eluted from kidneys with such lesions and labeled with fluorescein isothiocyanate. It was shown that these fluorescein-labeled eluates reacted with the corresponding antigens in the tubular deposits and also with the antigens present in the brush border and/or cytoplasm of the proximal tubules. The antigens are found in proximal tubules of the kidney but not in brain, lung, heart, liver, spleen, bowel, muscle, or urine. They appear to be soluble but may also be present in the plasma membrane. (Am J Pathol 88:135–144, 1977)

PREVIOUS STUDIES have shown that rabbits injected with nonglomerular components of rabbit kidney incorporated in Freund's complete adjuvant develop a lesion characterized by extensive interstitial fibrosis, tubular degenerative changes, and sparse focal lymphocytic infiltrates and by the deposition of IgG and C3 in a granular pattern along the basement membrane of the proximal convoluted tubules.[1,2] The immunization also resulted in the production of antibodies with several different specificities, including at least three kidney-specific autoantibodies.[2-4] When sera from animals with the tubular lesions were layered on normal rabbit kidneys, binding of IgG to the cytoplasm of proximal tubules could be demonstrated. Similar deposits could be produced in normal kidneys *in vivo* by passive transfer with serum. Transplantation of normal kidneys into rabbits with tubular lesions resulted in recurrence of the lesions in the graft in 3 of 6 animals.[5] Animals with severe disease developed renal glucosuria and generalized aminoaciduria.[6] The pathogenetic mechanism for this lesion is most probably the following: as antigen "leaks" out of the proximal tubule it reacts with the corresponding antibody in the perivascular spaces, forming a local immune complex.[2]

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It is the purpose of this paper to characterize further the antibodies eluted from kidneys with the tubular lesion.

Materials and Methods

Sera and kidneys used in this study were obtained from rabbits used in experiments described previously.[2] A total of 13 rabbits were repeatedly injected intradermally with 1 ml of a homologous kidney suspension incorporated in an equal volume of complete Freund's adjuvant, and 5 rabbits were injected with homologous liver suspension. In addition, 8 other rabbits were injected in a similar manner with the following antigens: a) 3 rabbits with 1 ml urine obtained from normal rabbits or from animals with the tubular lesions, concentrated ten times; b) 2 rabbits with 1 ml of rabbit kidney extract prepared as previously described;[2] and c) 4 rabbits with rabbit kidney cell membranes prepared according to the method of Edebo *et al.*[7] Briefly, the kidneys were minced, rinsed with phosphate-buffered saline (PBS), pH 7.3, and then homogenized in a medium of 0.25 M sucrose, 0.001 M $NaHCO_3$ and 0.0002 M $MgCl_2$. The homogenate was then centrifuged at 750*g* for 10 minutes and washed twice in the same medium. The sediment was then washed twice in 0.01 M $NaHCO_3$, once in 1 M NaCl before being suspended in 2.7 M KBr. After centrifugation at 35,000*g* for 60 minutes, the flotsam was harvested.

The rabbits were given a total of eight or ten injections at 2-week intervals and were biopsied 2 weeks after the last injection.

Elution of antibodies was carried out as follows: the kidneys were minced with scissors, washed with 0.15 M saline, and then homogenized at 4 C using a Potter Elvehjem tissue grinder. The suspension was centrifuged at 1100*g*, and the sediment was washed four to six times with 0.15 M saline. Citrate buffer (0.02 M, pH 3.2) was then added to the sediment to make an approximate 10% suspension. After incubation at 37 C for 1 hour in a shaking waterbath, the suspension was centrifuged at 80,000*g* for 30 minutes. The pH of the supernatant was adjusted to 7.2 with NaOH. It was then dialysed against PBS, pH 7.3, and concentrated two- to fourfold.

The kidneys of 4 rabbits that had been immunized with liver and did not have tubular deposits were pooled, and an acid eluate was prepared. Similarly, the kidneys of 5 rabbits that had been immunized with kidney and which had deposits were pooled and also eluted. Individual kidneys of 5 immunized rabbits were also eluted (Table 1).

Protein concentrations of the eluates were determined using the biuret method and the IgG concentrations by means of radial immunodiffusion.[8] Techniques and reagents used for immunofluorescence and serologic tests have been previously described.[2] Blocking tests were performed by layering the test serum on the kidney section, incubating for 1 hour at room temperature, washing for 20 minutes, and then applying the fluorescein-labeled eluate. Some sera were aggregated by heating at 63 C for 10 minutes before they were used in the blocking test. The following additional tissues were studied by the indirect immunofluorescence technique: brain, lung, heart, liver, spleen, jejunum, colon, and muscle.

Results

Most of the rabbits that were injected with an homologous kidney suspension incorporated in Freund's complete adjuvant developed a lesion characterized by extensive interstitial fibrosis, tubular damage, and scanty lymphocytic infiltrates and by granular deposits of IgG and C3 along the basement membranes of proximal tubules. All rabbits injected in a similar manner with homologous kidney extract or with homologous plasma membranes of renal tubules developed similar but milder lesions. Rabbits injected with homologous liver or with concentrated rabbit urine failed to develop such renal lesions.

The elution procedure was carried out at the same time on all kidneys except on those of Rabbit 2894. The amount of protein in the eluates from kidneys with the tubular deposits in all cases was greater than in the eluates from kidneys without such deposits (Table 1). Furthermore, only 11% of the protein in the eluate from kidneys of rabbits immunized with liver was IgG, while in the eluate from kidneys of rabbits with tubular deposits, 23% of the protein was IgG. When the eluates were tested in double diffusion gel precipitation against an antiserum to rabbit IgG, a reaction of identity was obtained with the eluate from the kidneys of rabbits immunized with kidney, the eluate from the kidneys of rabbits immunized with liver, and normal rabbit serum (Figure 1). When eluates were tested against an antiserum to rabbit serum, only one precipitation line was obtained (Figure 2). When the eluates were examined for antibody activity in the tanned cell hemaglutination and complement fixation tests using extracts of kidneys as well as other organs, negative results were obtained.

The eluates from individual kidneys as well as the serum from 1 rabbit (No. 2894) were labeled with fluorescein isothiocyanate. The characteristics of these conjugates are shown in Table 2. These conjugates were applied to frozen sections of kidneys from rabbits which had been immunized with kidney or liver, and the results are shown in Table 3. It can be seen that whenever tubular deposits of IgG were present, tubular deposits could also be demonstrated using serum from a rabbit (No. 2894)

Table 1—Protein Concentration in the Eluates

Rabbit No.	Immunized with	Tubular deposits	Protein (mg/ml)*	IgG (mg/ml)†
3463, 3464, 3465, 3476	Liver	No	0.53	0.06
3467, 3469, 3473, 3474, 3475	Kidney	Yes	1.3	0.31
3464	Liver	No	0.9	ND
3467	Kidney	Yes	1.2	ND
3471	Kidney	Yes	1.1	ND
3472	Kidney	No	0.9	ND
2894	Kidney	Yes	1.4	ND

ND = not done.
* Determined using the biuret method.
† Determined in triplicate using radial immunodiffusion.

Table 2—Characteristics of Conjugates Used

Rabbit No.	Eluate from	Fluorescein/protein ratio (μg/mg)	Optimal dilution of conjugate for staining*
3464	Kidney	8.6	Negative even when undiluted
3467	Kidney	2.8	1:4
3471	Kidney	4.8	1:16
3472	Kidney	12.0	Negative even when undiluted
2894	Kidney	7.6	1:64
2894	Serum	5.2	1:32

* Determined by checkerboard titration of the conjugates on kidneys with tubular deposits.

as well as with eluates of kidneys of rabbits with deposits (Rabbits 2894, 3467, and 3471). However, eluates of kidneys from rabbits (3464 and 3472) which did not have tubular deposits failed to stain deposits even when used undiluted. Furthermore, those conjugates which stained the granular deposits always stained the adjacent cytoplasm of the proximal convoluted tubule as well (Figure 3). It should also be noted that eluates of kidneys from rabbits with deposits (Rabbits 3467 and 3471) produced staining of the deposits in the kidneys from which they had been eluted

The staining produced by the fluorescein-labeled serum (Rabbit 2894) on normal kidneys was very intense and involved all of the cytoplasm of

Table 3—Reaction of Fluorescein-Labeled Eluates and Sera With Kidneys From Rabbits Immunized With Kidney or Liver

Kidney from Rabbit No.	Immunized with	Anti-IgG serum	Serum from Rabbit No. 2894*	Eluates of kidneys from Rabbit No.				
				2894*	3472†	3467*	3471*	3464‡
6258	Kidney	3+	1+	2+	–	1+	2+	–
6262	Kidney	–	–	–	–	–	–	–
6264	Kidney	–	–	–	–	–	–	–
6265	Kidney	–	–	–	–	–	–	–
3467	Kidney	1+	1+	2+	–	1+	1+	–
3468	Kidney	±	±	±	–	–	–	–
3469	Kidney	2+	2+	3+	–	1+	1+	–
3470	Kidney	1+	1+	2+	–	±	1+	–
3471	Kidney	2+	1+	3+	–	±	1+	–
3472	Kidney	–	–	–	–	–	–	–
3473	Kidney	3+	2+	3+	–	±	2+	–
3474	Kidney	2+	1+	2+	–	–	2+	–
3475	Kidney	1+	±	2+	–	–	–	–
3464	Liver	–	–	–	–	–	–	–
3478	Liver	–	–	–	–	–	–	–

The reactions of fluorescein-labeled reagents with the tubular deposits was arbitrarily graded from – to 3+.

* Rabbits 2894, 3467, and 3471 were immunized with kidney and had tubular deposits.

† Rabbit 3472 was also immunized with kidney but did not have tubular deposits.

‡ Rabbit 3464 was immunized with liver and did not have tubular deposits.

the proximal tubules but not other portions of the nephron. The pattern of staining produced by fluorescein-labeled eluates (which stained the tubular deposits) differed in most cases in that there was brighter staining in the brush border region (Figures 3 and 4) and only faint cytoplasmic staining. However, with a few eluates only cytoplasmic staining was noted (Figure 5). It should be noted that pretreatment of the frozen sections (i.e., elution of IgG) was not necessary to demonstrate the antigen in the deposits.

In many cases, the sections were approximately 1 year old, but they gave similar results with respect to staining for antigen in the brush border and in the deposits as sections from recently obtained kidneys. In old sections of some kidneys there was, however, marked loss of staining for IgG as well as for antigen even in the cytoplasm. In no instance did any of the fluorescein-labeled conjugates stain basement membranes of the tubules or glomeruli. The fluorescein-labeled material prepared from eluates of kidneys that did not have deposits failed to stain the deposits and also failed to produce cytoplasmic staining. The staining of the antigen in the deposits and in the brush border produced by the eluates could be blocked by immune serum from animals with tubular lesions but not by normal rabbit serum, heat-aggregated normal rabbit serum, or serum from rabbits immunized with liver.

The fluorescein-labeled eluates and serum were also tested on other rabbit tissues. As expected, the serum contained non-organ-specific autoantibodies which reacted with many other tissues such as liver. However, none of the eluates reacted with tissues of any organ other than kidney.

To determine the character of the antigen, rabbits were immunized with rabbit kidney extract which contained only saline-soluble constituents, with pooled rabbit urine both from normal animals and animals with tubular deposits, and with plasma membranes prepared from rabbit kidney. It can be seen in Table 4 that immunization with extract and plasma membrane did induce tubular deposits whereas immunization with urine did not.

Discussion

Using the technique of acid elution, it has been shown that IgG could be eluted from kidneys with deposits of IgG and C3 along the basement membrane of the proximal tubules. When the γ-globulin fraction of the eluted material was labeled with fluorescein isothiocyanate, it could be shown to stain the tubular deposits as well as the cytoplasm of the adjacent tubular cells. The reactivity of the conjugates could not be

Table 4—Immunofluorescent Findings in the Kidneys of Rabbits Immunized With Different Renal Antigens

Rabbit No.	No. of injections	Antigen	Tubular deposits
6236	8	Kidney extract	1+
6238	8		3+
6270	10	Concentrated pooled	−
6271	10	rabbit urine	−
6272	10		−
6232	8	Plasma membrane	1+
6234	8		1+
6268	8		1+

blocked by normal rabbit serum, heat-aggregated normal rabbit serum, or serum from animals immunized with liver but could be blocked by immune serum from rabbits with tubular deposits, including the animal's own immune serum. Heat-aggregated serum was used to exclude the possibility that rheumatoid-like factor activity was being demonstrated, since most rabbits immunized with kidney develop 7S antigammaglobulin factors. Thus, the inability of the heat-aggregated serum to block the reactivity of the conjugates as well as the reaction of the conjugates with the cytoplasm of proximal tubules suggests that there is antibody activity in the eluates which detects the antigen in the deposits and also in the cytoplasm of the proximal tubules. The fact that two different patterns of staining were noted suggests that perhaps there are two different antigens which are involved in the deposits. This is compatible with the hypothesis that the deposits represent antigen "leaking" out of the proximal tubules which has reacted with its corresponding antibody in the interstitium.[2] The fact that the eluates reacted only with kidney but not other organs is in keeping with the observation that the lesion can be induced by immunization with kidney but not liver. It was noted that antigen, antibody, and C3 could no longer be detected in basement membranes of the proximal tubules of some kidneys after prolonged storage at −20 C. Since this happened only with some kidneys, it suggests that at least one of the antigens may be labile under these conditions.

The experiments in which kidney extract, plasma membranes, and urine were used for immunization suggests that either the antigen is absent from urine or it is degraded into nonimmunogenic fragments. The ability of both a soluble fraction and an insoluble fraction (plasma membrane) to induce the tubular lesions could be explained by the fact that the soluble component was absorbed to the plasma membrane. Results of absorption studies carried out previously are in keeping with this.[2]

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[*Illustrations follow*]

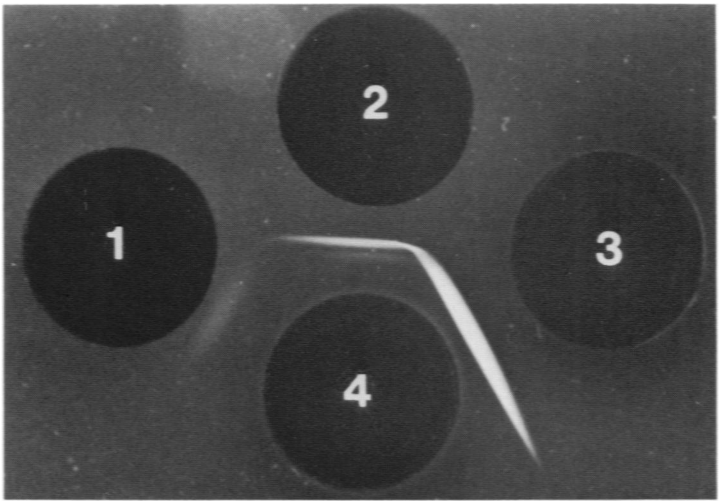


Figure 1—Double diffusion gel precipitation test. *Well 1,* eluate from kidneys of rabbits immunized with liver; *Well 2,* eluate from kidneys of rabbits immunized with kidney; *Well 3,* normal rabbit serum; and *Well 4,* goat antiserum to rabbit IgG. A reaction of identity can be seen.

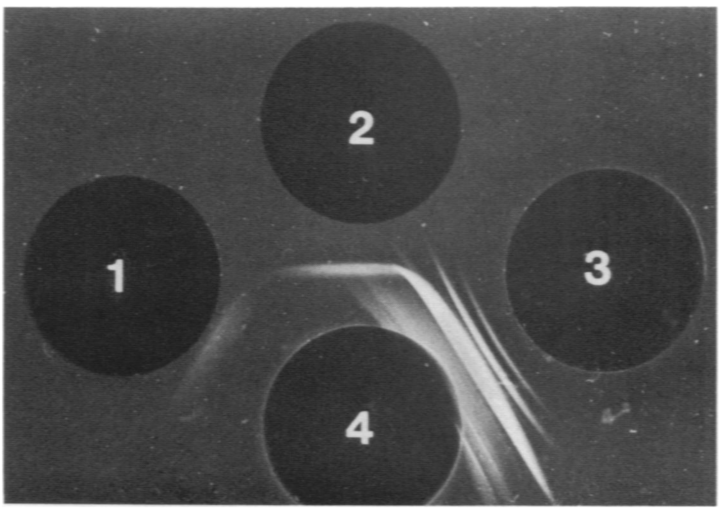
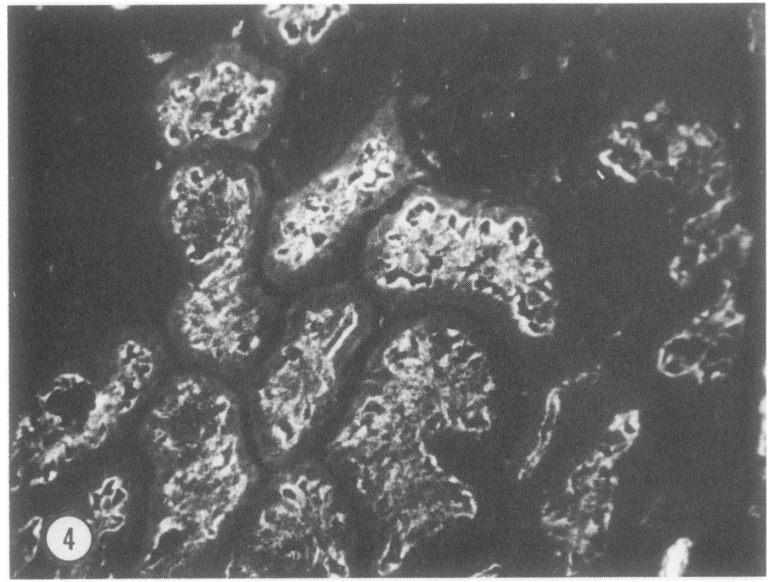


Figure 2—Double diffusion gel precipitation test. *Well 1, 2, 3,* same as in Figure 1; *Well 4,* goat antiserum to rabbit serum. Multiple precipitation lines have formed between the antiserum and the normal rabbit serum. One of these lines forms a reaction of identity with that produced by the eluates in Wells 1 and 2.



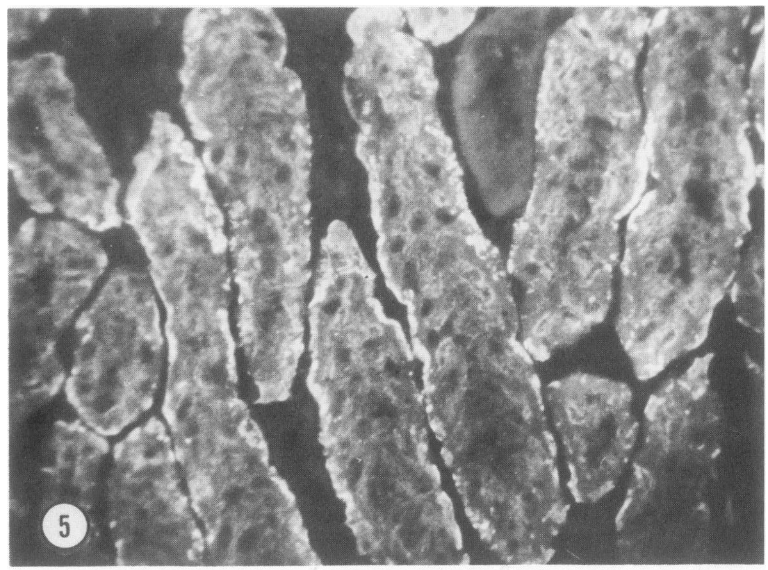


Figure 3—Section of a kidney from Rabbit 3471 stained with the fluorescein-labeled eluate from the kidneys of Rabbit 2894. Staining of the deposits along the tubular basement membranes as well as staining of the brush border of the proximal tubular cells can be seen. (× 200) **Figure 4**—Section of a normal rabbit kidney stained with the fluorescein-labeled eluate from the kidney of Rabbit 2894. Staining of only the brush border of proximal tubular cells can be seen. (× 200) **Figure 5**—Section of a kidney from Rabbit 3473 stained with the fluorescein-labeled eluate from the kidney of Rabbit 3471. Staining of the deposits along the tubular basement membrane as well as diffuse staining of the cytoplasm of the proximal tubular cells can be seen. (× 200)