Nonglomerular Renal Disease Produced in Rabbits by Immunization with Homologous Kidney

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IT HAS LONG BEEN KNOWN that the glomerulus is susceptible to immunologic injury. Two major forms of experimental glomerulonephritis can be produced by immunologic means.[1,2] In one form, antibodies elicited by immunization with autologous, homologous or heterologous glomerular basement membrane react with the corresponding antigens in the glomeruli of the host, initiating a sequence of events leading to glomerulonephritis.[3,4] In the other model, the damage is elicited by immune complexes deposited in the glomeruli from the circulation. The antigen participating in the formation of the complexes may be either endogenous [5] or exogenous.[6]

Little attention has been paid to the possibility that renal structures other than the glomeruli can be damaged by immunologic mechanisms. However, in both types of experimental glomerulonephritis, nonglomerular lesions—namely, interstitial infiltrates of mononuclear cells and tubular degenerative changes, have been reported.[1,7] The question of whether or not the nonglomerular lesions were secondary to glomerular damage remained unanswered, and the pathogenesis of the lesions was not clarified in these studies. In another study, Unanue *et al* [4] showed that some rabbits immunized with homologous renal tissue developed not only glomerulonephritis, but also tubular lesions that were characterized by deposits of immunoglobulin and complement along the tubular basement membranes. These authors suggested that this type of lesion resulted from the reaction of autoantibodies with antigenic determinants of tubular basement membrane. In addition, Steblay [8] observed that guinea pigs immunized with heterologous renal tissue developed acute tubular necrosis and a diffuse interstitial mononuclear cell infiltrate. The hypothesis that nonglomerular lesions may have an autoimmune basis also finds support in investigations showing that immunization of rabbits with homologous kidney suspensions in

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Freund's adjuvant resulted in the formation of kidney-specific autoantibodies.[9–11]

Previous studies in this laboratory on renal allografts have also provided evidence that interstitial and tubular disease could result from immunologic mechanisms.[12] It was shown that 3 of 11 rabbits that received two renal homografts developed kidney-specific antibodies as well as deposits of immunoglobulin and electron-dense deposits along the basement membrane of convoluted tubules. In continuing these studies, 1 rabbit was observed in which a renal graft functioned for 53 days. Histologic examination revealed interstitial fibrosis not only in the graft but also in the host's own kidneys. This led to the hypothesis that the destruction of the homograft could evoke an autoimmune response that damaged both the graft and the host kidneys.

Thus, several observations suggest that autoimmune mechanisms may lead to tubular and interstitial renal disease; however, this possibility has not been explored systematically. The purpose of the present study was to attempt to produce such lesions in rabbits by immunization with preparations of homologous kidney in Freund's adjuvant.

Materials and Methods

Rabbits. New Zealand white rabbits, weighing 2½ to 3 kg each, were used. Thirteen rabbits were immunized with rabbit kidney and another 5 with rabbit liver.

Antigens. Unpaired rabbit kidneys were obtained from Pel Freeze Co, Roza, Kansas. The cortices from 40 kidneys were minced and homogenized at 4 C in an equal volume of 0.15 M saline. The homogenate was centrifuged at 10,000 *g* for 20 minutes; the supernatant was used for immunization. To prepare extracts to be used for serologic tests, the homogenate was exposed to ultrasonic vibrations at 20,000 cycles/second for 3 minutes. The sediment was discarded after centrifugation at 105,000 *g* for 30 minutes. Pooled rabbit livers and other organs were processed in a similar manner.

Immunization Procedures

The antigens were emulsified with an equal volume of Freund's adjuvant (Difco Laboratories, Detroit, Michigan). The adjuvant used for the first four injections contained 8, 6, 4 and 2 mg of heat-killed *Mycobacterium tuberculosis* H37 Ra per ml, respectively; for the remaining injections, adjuvant without mycobacteria was used. Two milliliters of the emulsion were administered at each injection, intradermally in the back or in the foot pads. Four rabbits were given injections of the kidney antigen at intervals of 2 weeks for a total of ten injections. The other 9 rabbits immunized with kidney and those immunized with liver were started on the same schedule of injections but they were given a 4-month rest between the fifth and sixth injections. Bleedings were taken before each injection and at intervals after the end of the immunization schedule. Open renal biopsies were performed in 12 rabbits at 5, 7 or 11 weeks after the first injection and all animals were biopsied at the end of the immunization. The second biopsy was never taken from the same kidney as the first. All rabbits were killed within 4 months after the last injection, except for two rabbits immunized with kidney that were sacrificed at 13 and 18 months.

Serologic Procedures

Double Diffusion Gel Precipitation Test. This was performed in Noble agar or agarose at a concentration of 1.0% in 0.15 M NaCl. Plates were incubated at room temperature for 3–6 days.

Complement Fixation Test. A total volume of 0.5 ml was used, with all the reagents prepared in a volume of 0.1 ml. The sera were inactivated at 56 C for 30 minutes and used at a dilution of 1:15. Two 100% hemolytic units of guinea pig complement were employed. Fixation was carried out at 4 C for 18 hours, then at 37 C for 1 hour. After the addition of sheep erythrocytes sensitized by hemolysin in excess, the tubes were incubated at 37 C and readings taken at 30 minutes.

Mixed Agglutination Test. This was performed as previously described.[13] Monolayer cell cultures of lungs and kidneys were prepared from 12 normal rabbits. In most cases, the test was performed simultaneously with cultures of the lung and kidney from the same rabbit.

Tests for Antiglobulin Antibodies. These were performed as described in detail in a previous publication.[14]

Absorptions of Sera. To each milliliter of serum, which was diluted 1:10, 10 mg of lyophilized kidney or liver extract was added. The absorption was carried out with constant agitation at room temperature for 1 hour and at 4 C for 20 hours. The serum was withdrawn after centrifugation at 30,000 rpm for 30 minutes. Thereafter, the absorption procedure was repeated once.

To make tissue sediment for absorptions, the homogenate of kidney or liver was centrifuged at 10,000 *g* for 20 minutes. The supernatant was then centrifuged at 30,000 *g* for 30 minutes. The resulting sediment was washed until the washing fluid was quite clear. For each milliliter of serum diluted 1:10, 0.1 ml of sediment was used for absorption, which was carried out as described above.

Immunofluorescence. Goat antisera to rabbit IgG, IgA and IgM were kindly supplied by Dr. R. Genco of the Department of Oral Pathology of the School of Dentistry at the State University of New York at Buffalo. Goat antiserum to rabbit β1C was purchased from Cappel Laboratories, Downington, Pennsylvania. The antisera were monospecific, as demonstrated by double diffusion in gel and by immunoelectrophoresis. Goat antiserum to rabbit albumin, purchased from Rockland Laboratories, Gilbertsville, Pennsylvania, reacted with albumin and with another serum component having an α2 mobility, probably α2 macroglobulin. Guinea pig anti-rabbit fibrinogen serum was obtained as previously described.[15] Antiserum to rabbit C1q was made by immunizing rats with heat-aggregated rat γ-globulin that had been incubated with fresh rabbit serum. The antiserum was absorbed with heat-inactivated rabbit serum to render it monospecific. All antisera were conjugated according to the method of Beutner *et al.*[16] The ratios of fluorescein to protein ranged from 2.2 to 3.8. The conjugated sera gave no reactions with normal rabbit tissues. Additional evidence of the specificity of the anti-IgA, IgG and IgM sera was provided by demonstrating different patterns of reactivity in sections of diseased rabbit kidneys stained for only one of the immunoglobulins.

Fresh tissue obtained by biopsy or at autopsy was frozen in liquid nitrogen and stored at −20 C. Sections were cut at 4 μ. Two methods were used. (1) The stain to determine which serum components had bound to the kidney *in vivo* was made by layering the appropriate fluorescein-conjugated antisera on air-dried sections for 30 minutes. After washing for 30 minutes in phosphate-buffered saline, pH 7.3, the sections were mounted in 50% glycerol. (2) The stain to determine the presence of circulating immunoglobulins having the capacity to bind to kidney tissue *in vitro* was made by layering the antisera on air-dried sections of normal rabbit kidney. After 30 minutes of incubation at room temperature, the sections were washed and then stained with conjugated goat anti-rabbit IgG, IgA and IgM. These

Table 1. Summary of Serologic and Pathologic Findings in Rabbits Immunized with Homologous Kidney and Liver

Rabbit No.	Double diffusion gel precipitation test			Complement fixation test with extracts of		Pathology*		Deposition of globulin and complement **in vivo** in	
	Kidney-specific antibodies	Liver-specific antibodies	Non-organ-specific antibodies	Kidney	Liver	Glomerular	Non-glomerular	Glomeruli	Tubules
Rabbits Immunized with Kidney									
2891†	+	—	+	+	+	—	+++	+	++
2892	+	—	+	+	+	—	+	—	+
2893†	+	—	+	+	+	+	+++	+	+++
2894	+	—	+	+	+	—	++	—	+++
3467	+	—	+	+	—	—	+++	—	++++
3468	+	—	+	+	—	—	±	—	+
3469	+	—	+	+	—	—	+++	—	+++
3470	+	—	+	+	—	+	+++	+	++
3471	+	—	+	+	—	+	++	+	++
3472	+	—	+	+	+	+	—	+	—
3473	—	—	+	—	—	—	+	—	++
3474	+	—	+	+	+	—	±	—	++
3475	+	—	+	+	+	—	+	—	+++
Rabbits Immunized with Liver									
3463	—	+	+	+	+	—	—	—	—
3464	—	+	+	+	+	—	—	—	—
3465	—	—	—	—	—	—	—	—	—
3476	—	+	—	—	+	—	—	—	—
3478	—	+	+	—	+	+	—	+	—

* The extent of tubular cell changes and interstitial fibrosis was assessed by light microscopy and was graded —, ±, +, ++, +++.
† These 2 rabbits developed amyloidosis.

tests were performed with sera of rabbits immunized with kidney or liver. For comparative study, an antiserum with specificity for tubular basement membranes was employed. This antiserum was obtained from a rabbit that had received two kidney homografts from unrelated donors. Both rejected grafts showed, in addition to mononuclear cell infiltration, binding of IgG *in vivo* to the basement membranes of the convoluted tubules. When the serum from this rabbit or eluates from the renal grafts were layered on sections from 12 different normal rabbit kidneys that were then treated with a fluorescein-labeled goat antiserum to rabbit IgG, the basement membranes of the proximal tubules were stained in all rabbit kidneys tested. In none were the glomerular basement membranes stained.

Histology

Tissue to be processed for histology was fixed in 10% buffered formalin. Sections were routinely stained with hematoxylin and eosin, Masson trichrome, and with the periodic acid-Schiff method. Selected sections were also stained with phosphotungstic acid hematoxylin, silver methenamine, azancarmine, Sirius red, crystal violet thioflavin T and Congo red. Some frozen sections were also examined with a polarizing microscope.

Clinical Chemistry

For urine collections, the animals were housed in metabolic cages and allowed food and water *ad libitum*. Protein was determined according to the method of Shevky and Stafford.[17] Blood specimens were taken in the morning from nonfasting animals. Glucose in serum and urine was determined with an autoanalyzer in the clinical chemistry laboratories at the Buffalo General Hospital. Glucosuria was also detected by using Clinistix (Ames Co, Elkhart, Indiana).

Passive Transfer Experiments

Sera were obtained by extensive bleedings 1–3 months after the last injection of kidney or liver preparations and by exsanguinating the animals at the time of sacrifice. Ten to 20 ml of these sera were infused intravenously over a period of 1½ to 2½ hours every second or third day; a total of 70–90 ml was administered to each rabbit. Two days after the last infusion, one kidney was removed and 100 days later the rabbits were sacrificed.

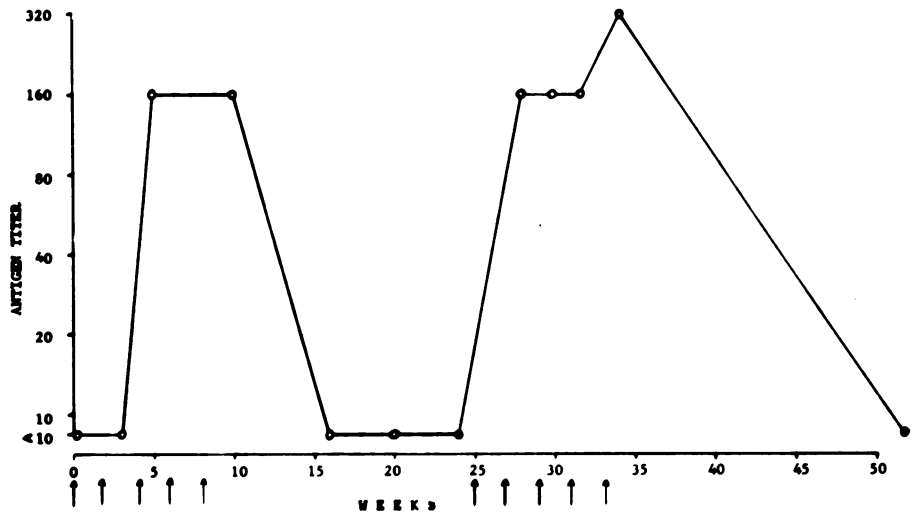
Results

Serology

The immunization of rabbits with homologous kidney or liver suspensions in complete Freund's adjuvant induced several different antibodies, as detected by the double diffusion gel precipitation test and using as antigens extracts of rabbit kidney, liver, heart and lung (Fig 1A, B and C). At least three kidney-specific, two liver-specific, and three non-organ-specific antibodies were demonstrated. Table 1 shows that, of the 13 rabbits immunized with kidney, 12 had kidney-specific antibodies and all 13 had non-organ-specific antibodies. Of the 5 rabbits immunized with liver, none had kidney-specific antibodies, 4 had liver-specific antibodies and 3 had non-organ-specific antibodies. The non-organ-specific

antibodies reacted with heart and lung extracts in addition to extracts of kidney and liver. In several instances, reactions between serum and organ extracts originating from the same animal were demonstrated, which points to the autoantibody nature of the antibodies. Two sera from animals injected with liver had one or two serum isoprecipitins (Fig 2). The identity of the serum isoantigens (allotypes) detected by these antibodies was not ascertained.

Of the 13 rabbits immunized with kidney, 12 formed antibodies that reacted with extract of rabbit kidney in the complement fixation test, while 2 of 5 rabbits immunized with liver had antibodies with similar reactivity. Complement fixation reaction with liver extract was exhibited by sera of 7 rabbits immunized with kidney and 4 rabbits immunized with liver. In general, the complement-fixing antibodies reacting with kidney and liver extracts tended to disappear after immunization was discontinued. Text-fig 1 shows the results of testing sequential sera



TEXT-FIG 1—Complement fixation tests with sequential serum samples from rabbit 3471 immunized with kidney. Titer is expressed as a reciprocal of highest dilution of rabbit kidney extract with which complete or almost complete inhibition of hemolysis was observed. *Arrows* indicate time of injections.

of rabbit 3471 against rabbit kidney extract. It can be seen that antibodies could be detected after the third injection (5 weeks after the first injection). When the immunization was interrupted after the fifth injection, the reaction disappeared, but it rapidly reappeared after immunization was resumed.

In addition, all the rabbits developed transplantation antibodies, as

demonstrated by the reaction with primary cell cultures of rabbit organs. Table 2 summarizes the results of mixed agglutination tests, with kidney and lung cultures from 6 rabbits exhibiting 43 positive reactions (titer of 90 or more) with kidney and/or lung. In 25 instances, the titer was significantly higher (nine times or more) with the kidney cell culture, while in only three instances was the titer significantly higher with the lung cell culture. In several instances, the serum of an immunized rabbit was tested against primary cell cultures of its own kidney or lung. Consistently negative results were obtained in these tests.

The sera from 14 of 18 rabbits agglutinated human Rh-positive erythrocytes sensitized by human incomplete anti-Rh antibodies. Negative results were obtained with sheep erythrocytes sensitized by rabbit antibodies. The agglutination of erythrocytes sensitized by human antibodies could be inhibited by Cohn fraction II of human, but not of rabbit, sera. The titer of agglutination, which ranged from 32 to 1024, was not affected by prior treatment of the serum with 2-mercaptoethanol. In double diffusion gel precipitation, tanned cell hemagglutination, and complement fixation tests, positive results were obtained with human but not with rabbit fraction II.

Histology

Renal biopsies were performed in 9 of the 13 rabbits immunized with kidney at 5, 7 or 11 weeks after the first injection. No glomerular or vascular abnormalities were found. However, in 2 rabbits there was very mild, irregular interstitial fibrosis and focal interstitial infiltration of mononuclear cells, which was predominantly lymphocytic. Questionable focal thickening of tubular basement membranes was also noted.

After 10 injections, 10 of 13 rabbits had moderate, irregular interstitial fibrosis mainly in the cortex, generally accompanied by sparse mononuclear cell infiltrates (Fig 3). The infiltrates were composed principally of lymphocytes, but in several kidneys small numbers of plasma cells were also noted. Definite tubular damage was now present in most kidneys, and was manifested by hyperchromatic nuclei, vacuolated and basophilic cytoplasm or loss of brush borders. In 4 rabbits, extensive tubular atrophy and dilation as well as occasional casts were seen. The tubular basement membrane was definitely thickened in all kidneys. No glomerular changes were noted.

Examination of kidneys taken at the time of sacrifice, 2–4 months after the last injection, showed that interstitial fibrosis had become more extensive, in some cases even involving the medulla extensively. Degenerative changes of tubules were more pronounced and in several

Table 2. Titers in the Mixed Agglutination Tests with Primary Cell Cultures of Rabbit Organs

Serum from rabbit No.	Titer of antibodies											
	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4		Rabbit 5		Rabbit 6	
	Kidney	Lung	Kidney	Lung	Kidney	Lung	Kidney	Lung	Kidney	Lung	Kidney	Lung
Rabbits Immunized with Kidney												
3467	810	270	810	<90	<90	<90	<90	<90	2430	<90	<90	<90
3468	90	<90	810	90			2430	2430	2430	<90	810	90
3469	810	90	810	<90			810	<90	2430	<90	<90	<90
3470	270	<90	2430	270	810	270	2430	810	810	<90	270	90
3471	<90	<90	270	<90	<90	<90	<90	<90				
3472	90	<90	<90	<90	90	<90	<90	270	810	<90	<90	<90
3473	810	810	810	<90								
3474	<90	270	810	90								
3475	270	2430	<90	<90								
Rabbits Immunized with Liver												
3463	810	810	2430	270	2430	270	2430	2430	2430	<90	2430	270
3464	270	810	270	<90	810	90	<90	<90	810	<90	90	<90
3476	270	<90	90	90								
3478	310	270	2430	90								
6052*	810	270					2430	2430	2430	270	2430	2430
37†	<90	<90	<90	<90	<90	<90	<90	<90	<90	<90	<90	<90

* This rabbit received four skin grafts.

† Normal rabbit serum.

cases the thickened tubular basement membranes appeared to be split (Fig 4 and 5). It was impossible to demonstrate deposits along the basement membranes of proximal tubules, using silver methanamine and azancarmine stains. In 4 rabbits, glomerular abnormalities were also present and consisted of slight endothelial and mesangial cell proliferation, as well as slight neutrophil infiltration. In 3 rabbits, the glomerular involvement was diffuse whereas in 1 rabbit it was focal (Fig 6 and 7).

Rabbits immunized with liver did not have any tubular or interstitial abnormalities even after a complete course of immunization. However, one of them had mild proliferative glomerulonephritis, again noted only at the time of sacrifice, 4 months after the last injection with liver. No pathologic changes were noted in the livers of animals immunized with either kidney or liver.

After 1 to 1½ years, two rabbits immunized with kidney developed amyloidosis involving mainly glomeruli, but with numerous small deposits also in the medulla and blood vessels (Fig. 8). The heart, adrenal, spleen, and liver of one rabbit were also involved. The presence of amyloid was confirmed by crystal violet, thioflavin T and Congo red stains and by use of the polarizing microscope.

Immunofluorescence Studies

In most of the rabbits immunized with kidney preparations, biopsy specimens revealed localization of IgG in a discontinuous pattern along the basement membrane of the proximal tubules. In some instances, only a small proportion of the tubules were involved and the deposits were small (Fig 9). However, in other instances, large amounts of IgG were present in many tubules. In some areas, the deposits looked like continuous band with irregular borders and all proximal tubules were involved (Fig 10A and B). However, this pattern was quite different from the thin continuous pattern produced by staining kidney sections with a rabbit antiserum specific for the basement membranes of proximal tubules (Fig 11) or from the binding of such antibody *in vivo* in the tubular basement membranes. When fewer than five injections had been given, only 2 of 6 rabbits had granular deposits of IgG along the proximal tubular basement membranes, and these were only focal. After ten injections, however, 10 of 13 rabbits had large deposits, 2 had focal deposits and in only 1 rabbit were no deposits seen. At this time, there was no localization of IgG in glomeruli or blood vessels. In the 4 months after the immunization was completed, the size and extent of the deposits increased in most rabbits, but in 2 cases in which very severe

parenchymal destruction occurred, most of the deposits became much smaller or disappeared.

The conjugated antisera to rabbit β1C and C1q gave staining patterns similar to that with anti-IgG. However, staining for IgA, IgM, albumin and fibrinogen gave consistently negative results. None of the rabbits immunized with liver had deposits of immunoglobulin or complement components along the tubular basement membranes.

Of the 12 rabbits with tubular deposition of IgG, 11 had kidney-specific antibodies detected by precipitation tests. Furthermore, these 11 rabbits also developed antibodies that fixed complement with extract of rabbit kidney, although only 7 of them exhibited complement-fixing antibodies when tested against extract of rabbit liver. One rabbit with tubular lesions had neither kidney-specific antibodies nor complement-fixing reactivity with kidney or liver extracts, while 1 rabbit had such antibodies but no tubular lesions.

When sera from the rabbits immunized with kidney or liver were layered on sections of normal rabbit kidney that were then stained for rabbit IgG, the cytoplasm of the proximal tubules was stained diffusely in all cases (Fig 12). Similar reactivity with antibodies of the IgA and IgM classes was not detected.

Absorption experiments are exemplified by the results presented in Table 3. Activity of anti-kidney sera was removed more efficiently by insoluble than by soluble preparations of kidney. It was not affected, however, by liver preparations. Anti-liver sera were successfully absorbed by soluble and insoluble preparations of both kidney and liver.

Four rabbits immunized with kidney and 1 rabbit immunized with liver had focal glomerular deposits of IgG and/or IgM as well as of β1C in mesangial regions. In one case, IgA was also seen in the deposits (Fig 13). These observations correlated well with the findings by light microscopy, since only the kidneys of these rabbits exhibited proliferative glomerulonephritis and only in the specimens taken 4 months after the end of the immunizations. The deposit of amyloid seen in 2 rabbits was shown to contain IgG and fibrinogen. Deposition of immunoglobulins or complement was not detected in the livers of 6 animals immunized with kidney and 3 immunized with liver.

Clinical Chemistry

Most rabbits immunized with kidney and liver had intermittent mild proteinuria (<50 mg/day). However, all 5 rabbits that developed glomerular abnormalities had more severe proteinuria, which reached values as high as 300 mg/day. The two rabbits with amyloidosis de-

Table 3. Reaction of Sera with Proximal Tublar Cytoplasm of a Normal Rabbit Kidney, as Detected by Indirect Immunofluorescence

Rabbit No.	Immunized with	Unabsorbed	Absorbed with extract of		Absorbed with sediment of	
			Kidney	Liver	Kidney	Liver
3469	Kidney	++	++	++	+	++
3471	Kidney	++	+	++	—	++
3475	Kidney	++	+	++	—	++
3463	Liver	+	—	—	—	—

veloped severe proteinuria with values of 300–1000 mg/day. Of the 13 rabbits immunized with kidney, 8 developed glucosuria even though they had normal levels of blood sugar. The glucosuria was noticed after eight or more injections. It was persistent in all 8 rabbits. None of the rabbits immunized with liver developed glucosuria.

Passive Transfer Experiments

Six rabbits were used as recipients; 4 rabbits were infused with a serum pool obtained from animals that had tubular deposition of IgG and complement, and 2 rabbits were infused with serum from rabbits immunized with liver (Table 4). No tubular abnormalities were observed by light microscopy in the kidneys of the recipient rabbits. However, the kidneys of the 4 rabbits infused with the serum pooled from rabbits with the tubular lesions were found to have the same pattern of immunofluorescent staining for IgG and β1C as the donors. The lesions were quite focal, however. Interestingly, the amount of IgG and β1C did not lessen appreciably during the 3-month observation period after transfer. In one kidney, there was also faint continuous localization of IgM but not β1C along the glomerular basement membrane. The re-

Table 4. Tubular Deposits in Rabbits After Passive Transfer with Serum

Serum Donor				
Rabbit No.	Immunized with	Recipient No.	Volume infused (ml)	Tubular lesion in recipient
Pool*	Kidney	6211	70	yes
Pool	Kidney	6212	70	yes
Pool	Kidney	6120	90	yes
Pool	Kidney	6123	90	yes
3463	Liver	6208	70	no
3464	Liver	6207	70	no

* The sera from rabbits 3467, 3470, 3471, 3473 and 3474 were pooled.

cipients of sera of rabbits immunized with liver had no tubular deposits. However, one of them had granular deposits of β1C but not immunoglobulins in mesangial regions. Rather large volumes of serum were used in these experiments, but it should be remembered that the sera were obtained 1–4 months after immunization—*ie*, at the time when their antibody activity was very low and, in many cases, not demonstrable.

Discussion

The present study has shown that immunization of rabbits with homologous kidney induced the formation of kidney-specific autoantibodies as well as renal damage involving tubules and interstitial tissue. The early renal lesion was characterized by deposition of IgG and complement components along the basement membranes of the proximal tubules. Similar findings were described by Unanue *et al*[4] in rabbits immunized with homologous kidney preparations, and these findings were found to be associated with a characteristic ultrastructural lesion with electron-dense deposits. Similar immunofluorescent and ultrastructural features have also been observed in rabbits that received repeated renal allografts.[12] Observation of the rabbits in the present study over prolonged periods after repeated injections with kidney revealed more severe renal damage, with tubular cell abnormalities, irregular, generally sparse mononuclear cell infiltration and interstitial fibrosis. Once initiated, the lesions appeared to be progressive. Functional tubular abnormalities evidenced by renal glucosuria also developed in many animals. The tubular deposits of immunoglobulin and complement could be induced in normal rabbits by transfer of immune serum.

Kidney-specific antibodies were found in the present study only in rabbits immunized with kidney. They exhibited at least three different specificities. Some rabbits immunized with liver formed liver-specific antibodies. Both groups of rabbits formed antibodies that reacted with an antigen (or antigens) present in kidney and liver extracts as well as in extracts of other rabbit organs. Since the renal tubular lesions were induced only by immunization with kidney, and since no histologic abnormalities or deposits of IgG or complement were noted in the livers of the immunized rabbits, the liver-specific and non-organ-specific antibodies did not appear to be of pathogenetic significance. Eleven of 12 rabbits in which IgG and complement components were deposited along the basement membrane of the proximal tubules had kidney-specific autoantibodies detectable with precipitation tests, while the 1 rabbit without deposits also had such antibodies. Similarly, all rabbits with

renal lesions except 1 had antibodies that fixed complement with extract of rabbit kidney. Indeed, sera of 5 rabbits reacted only with kidney but not liver extracts. However, it is not possible to draw any conclusions about the pathogenic role of these kidney-specific antibodies from these data.

On the other hand, it is likely that the IgG along the tubular basement membrane represented antibody combined with antigen rather than nonspecific accumulation of γ-globulin. For one thing, other plasma proteins (fibrinogen and albumin) were not present in the deposits. In addition, the complement components C1q and β1C were localized in the same deposits. The passive transfer experiments showed that the antibodies detected in the deposits were also present in the circulation.

The mode of formation of the deposits is, however, somewhat of an enigma at present. One possibility is that they originated from circulating antigen-antibody complexes, since the immunofluorescent staining occurred in a granular or beaded pattern, which is characteristic of deposits of immune complex in glomeruli. However, it does not seem likely that complexes passed through the glomeruli and then were reabsorbed intact, since in most cases no deposits were seen in glomeruli and since proteins undergo proteolysis when they are absorbed from the tubular lumen.[18] It does not appear likely either that complexes formed in the peritubular capillaries and were transferred across the basement membrane since, again, one would expect to see some complexes in the glomeruli or blood vessels. Another possibility is that the deposits were due to the reaction of antibody with antigens that are normal constituents of the tubular basement membranes, as suggested by Unanue *et al.*[4] However, as mentioned previously, the staining pattern did not resemble that produced *in vitro* or *in vivo* by antibodies specific for basement membranes of proximal tubules. For these reasons, the explanation that appears most likely is that the complexes were formed locally as a result of antigen "leaking" out of the cell and reacting with antibody as it diffused out of the peritubular capillary. Such a hypothesis is consistent with the immunofluorescence observations that sera of rabbits with the renal lesions reacted with the cytoplasm of the proximal tubules. Preliminary absorption studies indicated that rabbits immunized with kidney might have formed kidney-specific antibodies for cytoplasmic antigens. It should also be recalled that autoantibodies to kidney-specific antigens demonstrable by precipitation and complement fixation tests were formed only by rabbits immunized with kidney. Since it has not been determined that the specificity of the antibodies detected by these dif-

ferent technics is the same, it may be that several kidney-specific antibodies are involved. Whatever the nature of this lesion, however, it appears that it may eventually lead to interstitial fibrosis. Another abnormal finding consisted of focal mononuclear cell infiltrates, suggesting that delayed hypersensitivity may also be of pathogenetic significance.

It is of interest that a tubular lesion with ultrastructural features similar to those described in immunized rabbits [4] and in rabbits bearing renal allografts [12] has been described by Berger *et al* [19] in membranous glomerulonephritis in man. Furthermore, immunofluorescence findings of granular deposition of IgG and β1C that corresponded in localization to the electron-dense depostis along the basement membrane of proximal tubules seen ultrastructurally have been noted by Andres *et al* [20] in human renal allografts and by Klassen *et al* [21] in 3 cases of systemic lupus erythematosus and in a case of recurrent idiopathic hematuria. Thus, it is apparent that such tubular lesions occur also in man. It seems likely that they may have been overlooked in many instances because of fascination with the glomerular abnormalities. Furthermore, on the basis of the present findings, it is worth considering the possibility that various forms of renal disease characterized by unexplained interstitial fibrosis or mononuclear cell infiltration may involve an autoimmune mechanism.

That damage to the proximal tubule could result in functional abnormalities was anticipated. However, the vagaries of studies of renal function in rabbits [22] deterred us from doing more than preliminary studies. The occurrence of tubular dysfunction in renal grafts,[23] glomerulonephritis [24] and "nephrosis" [25,26] in human beings has been reported, but the nature of the pathologic changes responsible for this dysfunction has not been studied in detail. However, it may be that some of these patients had tubular lesions similar to those described in the present study.

All rabbits immunized with kidney and liver also developed antibodies to antigens of the rabbit cell surface (histocompatibility antigens), as detected with the mixed agglutination test. Many sera reacted more strongly with kidney than with lung cultures and some reacted only with kidney cultures. It should be noted that the cells in the primary cell culture of rabbit kidney look like epithelial cells, whereas those of rabbit lung resemble fibroblasts. This suggests that these sera may be recognizing antigens that are present in significantly higher density on epithelial cells. However, further tests with primary cell cultures of other organs and/or absorption studies with cells from various organs would be necessary to verify this. Since sera

of rabbits immunized with liver also exhibited such antibodies, and since these were isoantibodies rather than autoantibodies, it seems unlikely that they are of pathogenic significance with respect to the development of the tubular lesion.

Two rabbits immunized with liver also developed precipitins against two serum isoantigens (allotypes). In addition, antiglobulin antibodies were formed by most of the rabbits in both groups. The reactivity of these antibodies was directed against heterologous rather than homologous γ-globulin, which is similar to the reactivity of the antibodies produced by immunizing rabbits with denatured autologous γ-globulin.[14] There is no evidence for a pathogenic role for either of these two types of antibodies.

Assessment of the renal lesion in its early stages was difficult or impossible using only light microscopy, especially since the deposits could not be demonstrated satisfactorily using the silver methenamine or azancarmine stains. Later, interstitial fibrosis and tubular degenerative changes became apparent. However, the granular deposits of IgG and complement components detected by immunofluorescence became smaller and fewer in those kidneys where extensive tubular destruction and fibrosis had taken place. It seems likely that with time the deposits would disappear completely, leaving only a damaged kidney without clues to the underlying immunopathogenetic mechanism.

Four rabbits immunized with kidney and 1 rabbit immunized with liver developed glomerulonephritis, which was characterized by proliferation of mesangial and endothelial cells and accumulation of a few polymorphonuclear neutrophils. Immunofluorescence studies demonstrated immunoglobulins and β1C, but not albumin or fibrinogen, in granular clumps primarily in mesangial regions. Significantly, 1 of the rabbits immunized with kidney and the rabbit immunized with liver did not have any deposits along the tubular basement membranes. This glomerulonephritis was very likely due to a form of immune complex disease and unrelated to the tubular lesions. The absence of glomerulonephritis with "linear" staining, typical of anti-glomerular basement membrane disease, can be explained in the present study by the fact that glomeruli were removed from the immunizing preparation.

The finding of amyloidosis, associated with gross proteinuria, in 2 rabbits that were observed for many months after immunization was completed is not surprising in view of the well-known association between prolonged injections of various materials and amyloid.[27] However, the present studies provide no information as to a possible underlying immunologic mechanism.

Summary

Immunization of rabbits with suspensions of rabbit kidney incorporated in Freund's adjuvant induced renal abnormalities characterized by deposition of IgG and complement components along the basement membrane of the proximal tubules and by the development of interstitial fibrosis with minimal irregular infiltration of mononuclear cells. Abnormalities of tubular cells were seen histologically and were manifested functionally as renal glucosuria. Rabbits immunized with suspensions of rabbit liver did not develop these renal lesions.

Rabbits immunized with kidney suspensions developed kidney-specific autoantibodies detectable by precipitation and complement fixation tests. The antisera reacted with cytoplasmic constituents of the proximal tubules, as shown by immunofluorescence.

Transfer of serum from kidney-immunized donors to normal rabbits resulted in the appearance of deposits of IgG and β1C in the kidney in a pattern similar to that seen in the donors.

The hypothesis is advanced that the deposits are formed locally as a result of antigen "leaking" out of the tubular cells and reacting with antibody as it diffuses out of the peritubular capillary.

Some rabbits immunized with kidney or liver also developed glomerulonephritis which was apparently due to immune complexes. The nature of the antigen in the complexes has not been elucidated.

A variety of other antibodies were detected in sera of rabbits immunized with kidney or liver but none of these antibodies appeared to be of pathogenic significance.

References

1. Unanue ER, Dixon FJ: Experimental glomerulonephritis: immunological events and pathogenetic mechanisms. Advances Immun 6:1–90, 1967
2. McCluskey RT, Vassalli P: Experimental glomerular disease, The Kidney. Edited by C Rouiller, AF Muller. Vol II. New York, Academic Press Inc, 1969, pp 83–198
3. Steblay RW: Glomerulonephritis induced in sheep by injections of heterologous glomerular basement membrane and Freund's complete adjuvant. J Exp Med 116:253–272, 1962
4. Unanue ER, Dixon FJ, Feldman JD: Experimental allergic glomerulonephritis induced in the rabbit with homologous renal antigens. J Exp Med 125:163–175, 1966
5. Edgington TE, Glassock RJ, Dixon FJ: Autologous immune complex nephritis induced with renal tubular antigen. I. Identification and isolation of the pathogenetic antigen. J Exp Med 127:555–572, 1967
6. McCluskey RT: Experimental serum sickness, chap 20, The Inflammatory

Process. Edited by BW Zweifach, L Grant, RT McCluskey. New York, Academic Press, Inc, 1965, pp 649–683

7. Heymann W, Kmetec EP, Wilson SGF, Hunter JLP, Hackel DB, Okuda R, Cuppage F: Experimental autoimmune renal disease in rats. Ann NY Acad Sci 124:310–322, 1965
8. Steblay RW: Tubular necrosis induced in guinea pigs by injection of heterologous kidney antigens and Freund's adjuvant. Fed Proc 29:288, 1970
9. Milgrom F, Centeno E, Shulman S, Witebsky E: Autoantibodies resulting from immunization with kidney. Proc Soc Exp Biol Med 116:1009–1013, 1964
10. Centeno E, Shulman S, Milgrom F, Witebsky E, Skelton F: Studies on organ specificity. XVII. Autoantigens of rabbit kidney. J Immun 96:530–536, 1966
11. Shulman S, Wypych J: Kidney antigens active in isoimmunization. I. The antigenic diversity. Immunology 17:641–652, 1969
12. Klassen J, Milgrom F: Autoimmune concomitants of renal allografts. Transplantation Proc 1:605–608, 1969
13. McDonald JC, Milgrom F, Abeyounis CJ, Witebsky E: Mixed agglutination with cell cultures in rabbit homotransplantation. Proc Soc Exp Biol Med 118: 397–399, 1965
14. Milgrom F, Witebsky E: Studies on the rheumatoid and related serum factors. I. Autoimmunization of rabbits with gamma globulin. JAMA 174:56–63, 1960
15. Klassen J, Milgrom F: The generalized Shwartzman phenomenon in rabbits with denervated kidneys. Proc Soc Exp Biol Med 134:980–984, 1970
16. Beutner EH, Holborow EJ, Johnson GD: Quantitative studies of immunofluorescent staining. I. Analyses of mixed immunofluorescence. Immunology 12:327–337, 1967
17. Shevky MC, Stafford DD: Clinical method for estimation of protein in urine and other body fluids. Arch Intern Med 32:222–225, 1923
18. Wesson LG Jr: Proteins, Physiology of The Human Kidney. New York, Grune and Stratton, 1969, pp 246–251
19. Berger J, Galle P: Dépôts denses au sein des membranes basales du rein: Étude en microscopies optique et électronique. Presse Med 71:2351–2354, 1963
20. Andres GA, Accinni L, Hsu KC, Penn I, Porter KA, Rendall JM, Seegal BC, Starzl TE: Human renal transplants. III. Immunopathologic studies. Lab Invest 22:588–604, 1970
21. Klassen J, McCluskey RT, Milgrom F: An immunological renal tubular lesion in humans (unpublished data)
22. Smith H: Comparative physiology of the kidney, The Kidney. New York, Oxford University Press, 1951, pp 520–574
23. Györy AZ, Stewart JH, George CRP, Tiller DJ, Edwards KDG: Renal tubular acidosis, acidosis due to hyperkalaemia, hypercalcaemia, disordered citrate metabolism and other tubular dysfunctions following human renal transplantation. Quart J Med 38:231–254, 1969
24. Hawkings JR, MacKay EM, Van Slyke DD: Glucose excretion in Bright's disease. J Clin Invest 8:107–119, 1929
25. Stickler GB, Hayles AB, Power MH, Ulrich JA: Renal tubular dysfunction complicating the nephrotic syndrome. Pediatrics 26:75–85, 1960

26. Royer P, Delaitre R, Mathieu N, Gerbeaux S, Haib R, Koegel R: Le syndrome néphrotique avec insuffisance tubulaire globale et tétanie recidivante. Ann Pédiat 10:583–596, 1963

27. Schwartz P: New facts and concepts about causes and nature of amyloidosis, Amyloidosis: Cause and Manifestation of Senile Deterioration. Springfield, Illinois, Charles C Thomas, 1970, pp 18–19

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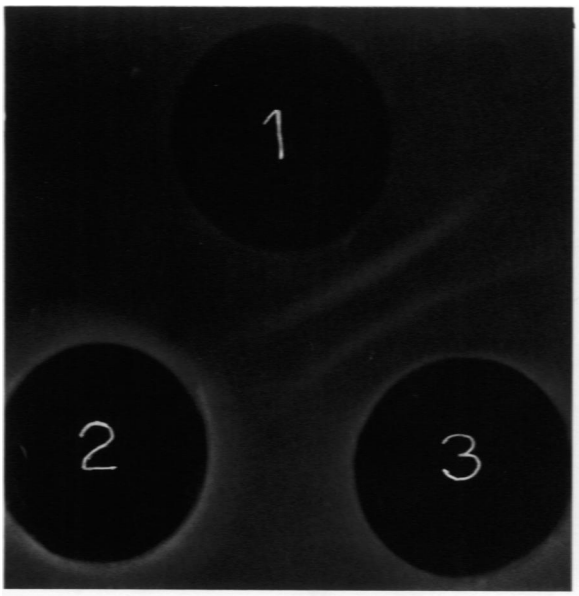


Fig 1A—Well 1: serum of rabbit 3467, immunized with kidney. Well 2: liver extract. Well 3: kidney extract. Two precipitin lines have formed with kidney extract but none with liver extract. A non-organ-specific antigen also was detected with other bleedings of this rabbit.

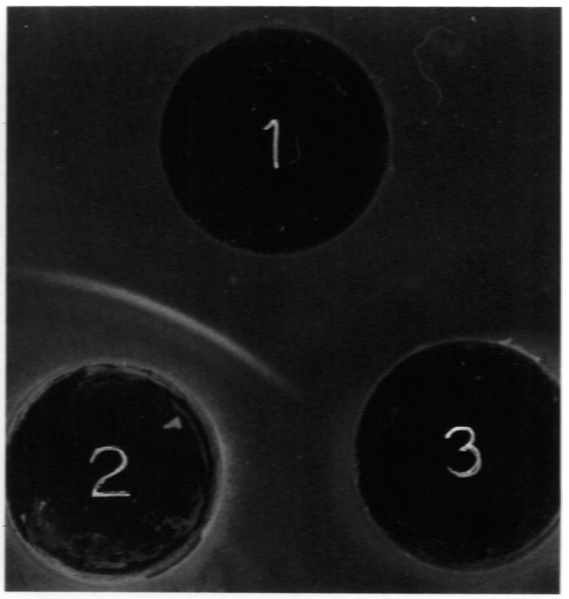


Fig 1B—Well 1: serum of rabbit 3478, immunized with liver. Well 2: liver extract. Well 3: kidney extract. Two precipitin lines have formed with liver extract but none with the kidney extract. A non-organ-specific antigen also was detected with other bleedings of this rabbit.

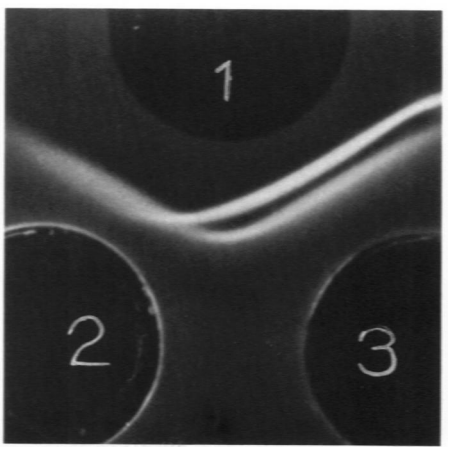


Fig 1C—Well 1: serum of rabbit 2893, immunized with kidney. Well 2: liver extract. Well 3: kidney extract. Two precipitin lines have formed with the kidney extract; both of them merge into identity reaction with line formed with liver extract. A kidney-specific antigen also was detected with other bleedings of this rabbit.

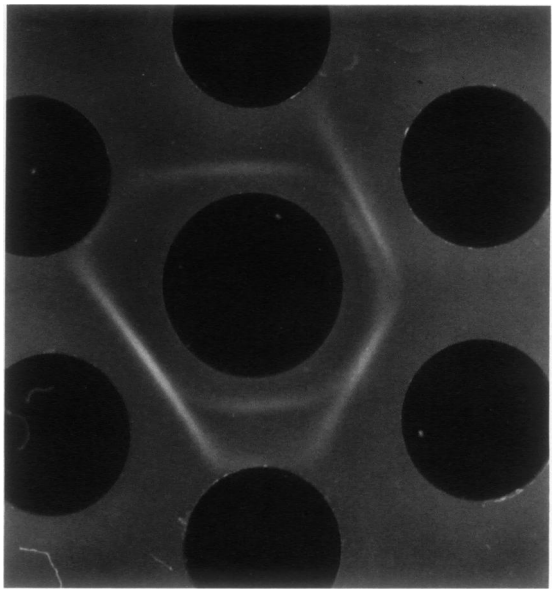


Fig 2—Center well: serum of rabbit 3464, immunized with liver. Peripheral wells: sera of 6 normal rabbits. Two distinct precipitin lines have formed.

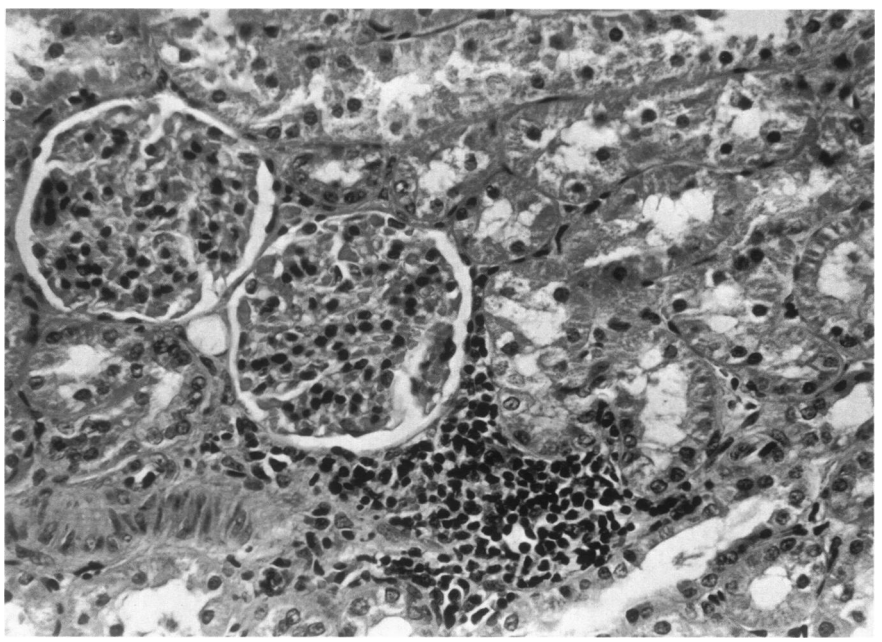
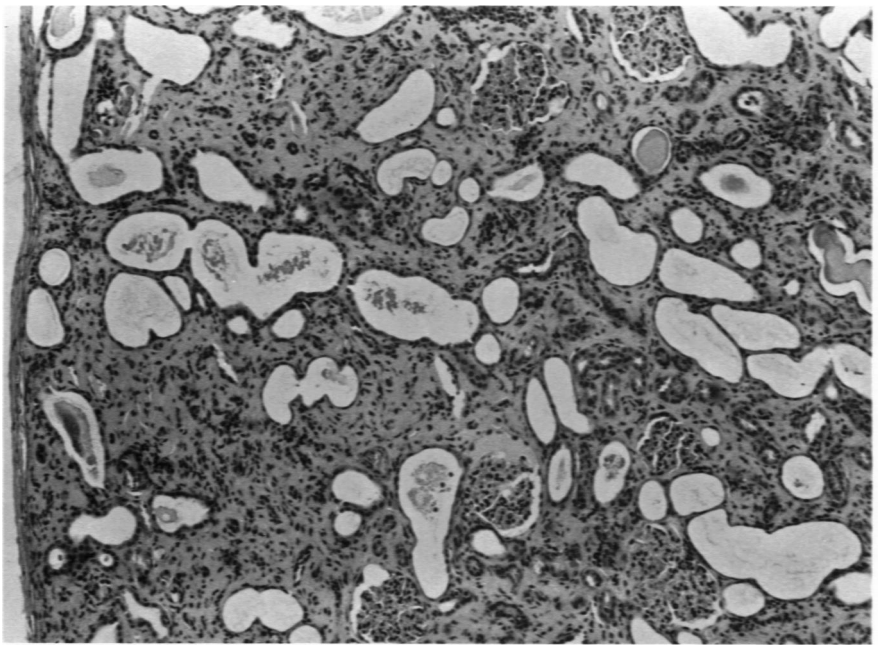


Fig 3—Section of a kidney from rabbit 3468 after ten injections with kidney. Small, focal infiltrate of mononuclear cells is seen (H&E, × 275).



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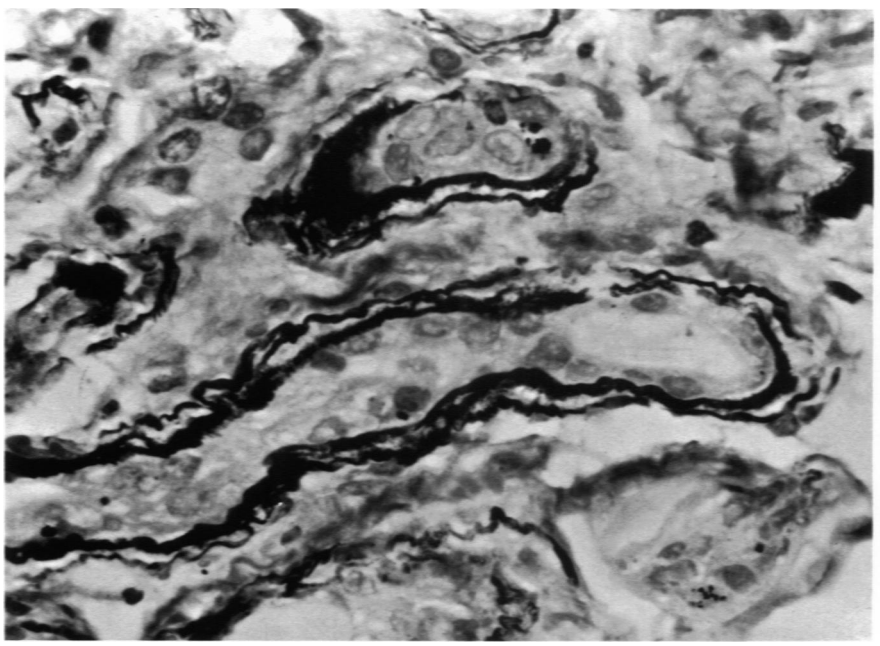

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Fig 4—Section of kidney from rabbit 3469 obtained 2 months after the last injection. Very severe interstitial fibrosis with a mild, focal infiltrate of mononuclear cells is seen. Severe tubular degenerative changes with irregular dilatation and atrophy are also present (H&E, × 76). **Fig 5**—Section of kidney from rabbit 3469 obtained 2 months after last injection. Reduplication of tubular basement membranes is seen (PAS-silver methenamine stain, × 680).

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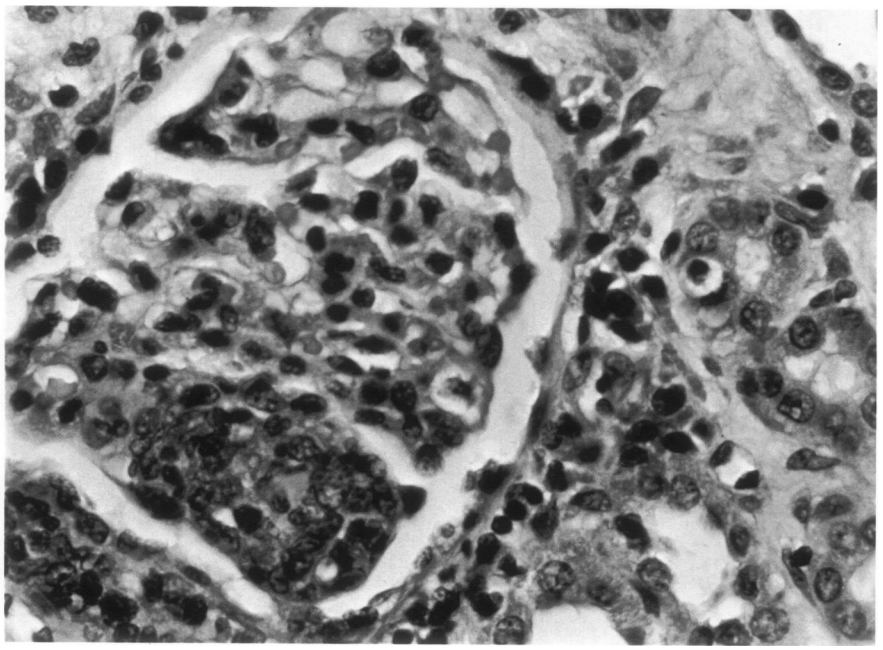

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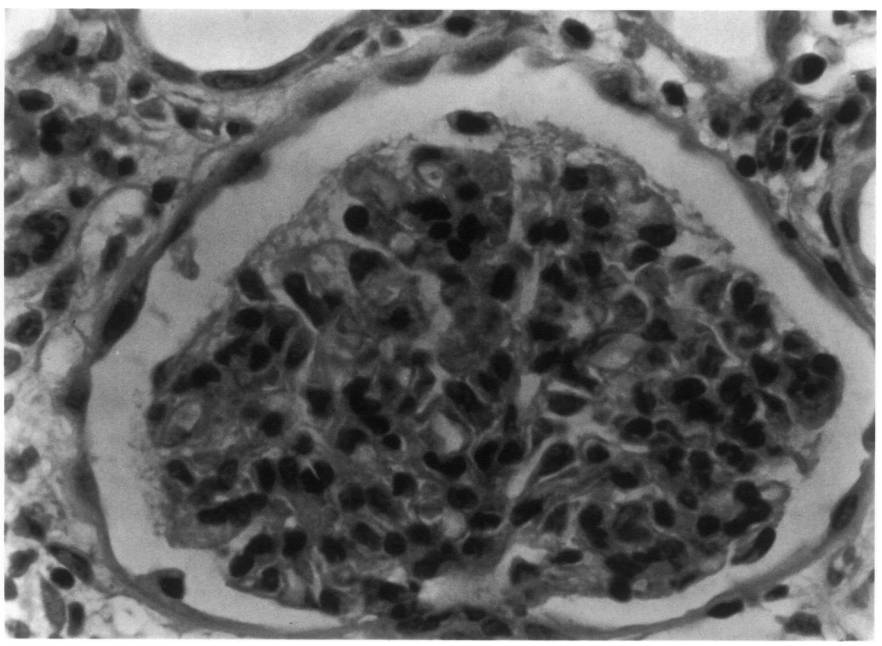
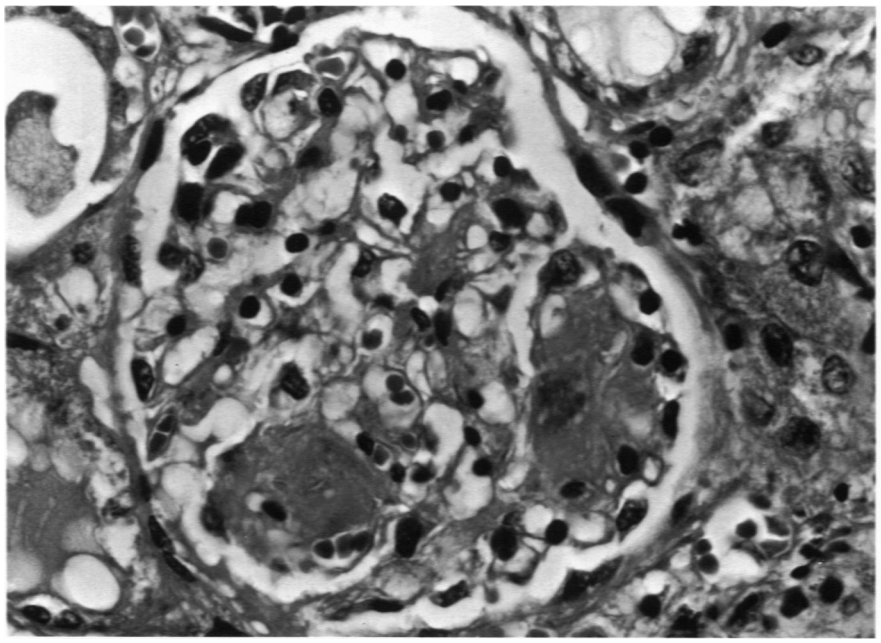


Fig 6—Section of kidney from rabbit 3471 obtained 4 months after last injection. Focal proliferative glomerulonephritis is seen (H&E, × 600). **Fig 7**—Section of kidney from rabbit 3470 obtained 4 months after last injection. Mild diffuse proliferative glomerulonephritis is present (H&E, × 600).



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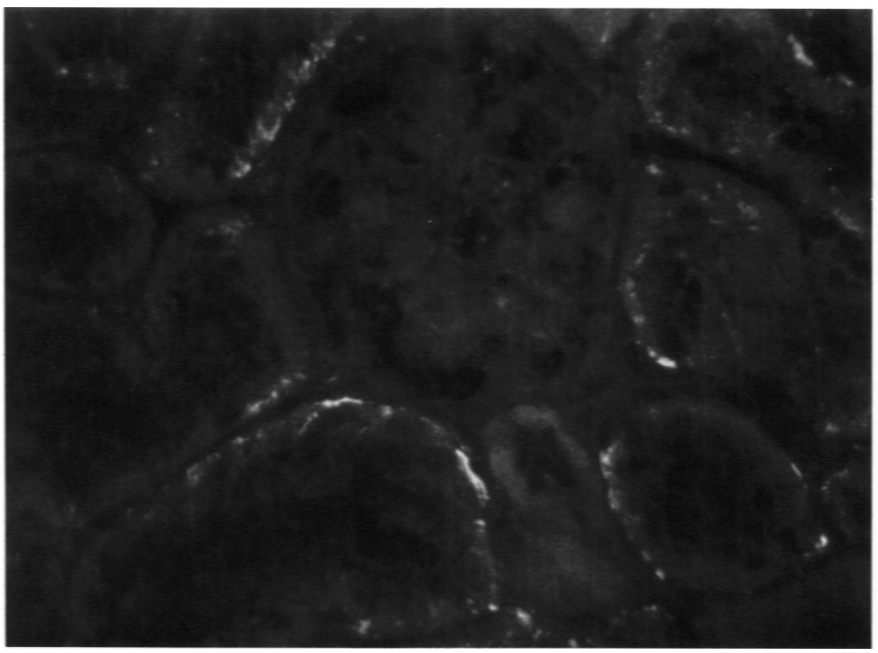

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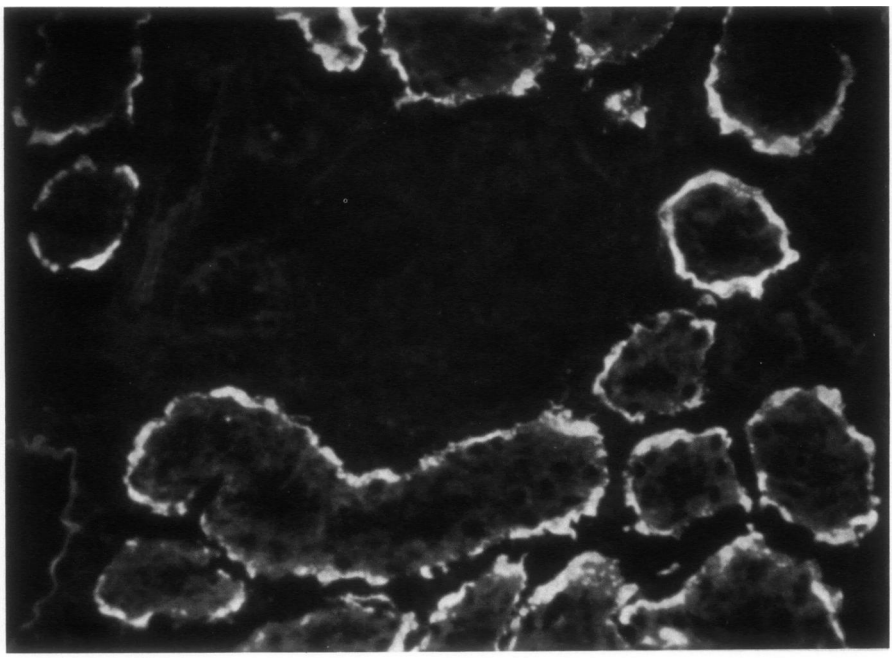
Fig 8—Section of kidney from rabbit 2891 at time of sacrifice. Large deposits of amyloid are seen in glomeruli (H&E, × 600). **Fig 9**—Section of kidney from rabbit 3468 obtained after ten injections with kidney and stained with a fluorescein-labeled antiserum to rabbit IgG. Focal granular deposits are seen along basement membranes of proximal convoluted tubules. This represents an early lesion (× 200).

10A



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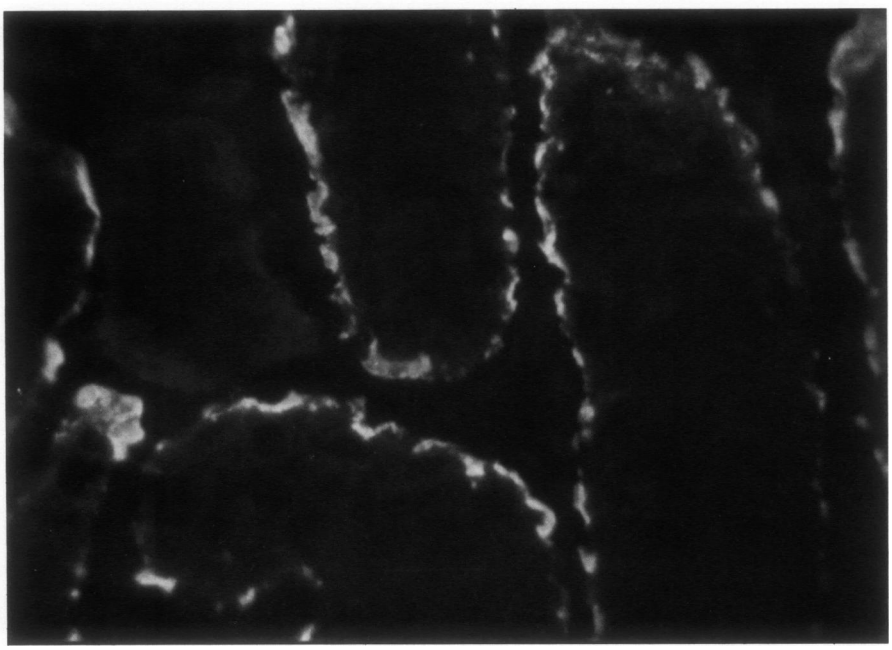
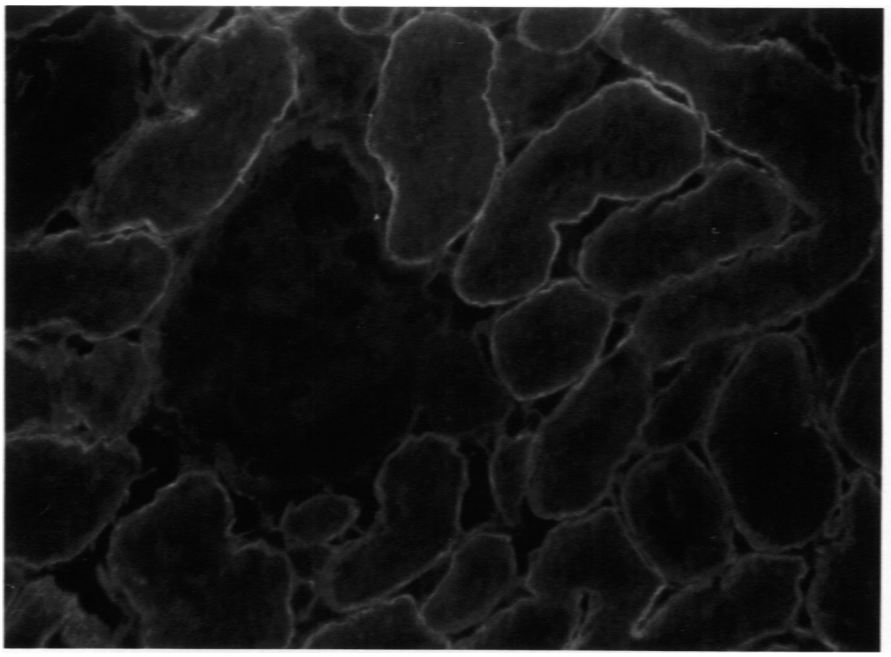


Fig 10A—Section of a kidney from rabbit 3475 obtained after ten injections with kidney and stained with fluorescein-labeled antiserum to rabbit IgG. Large granular deposits are seen along basement membranes of proximal tubules. No deposits are seen in glomerulus, blood vessels or distal convoluted tubules (× 200). **B**—Same section at higher magnification. Granular pattern of fluorescence is clearly seen along basement membrane of proximal, but not distal convoluted tubule (× 400).



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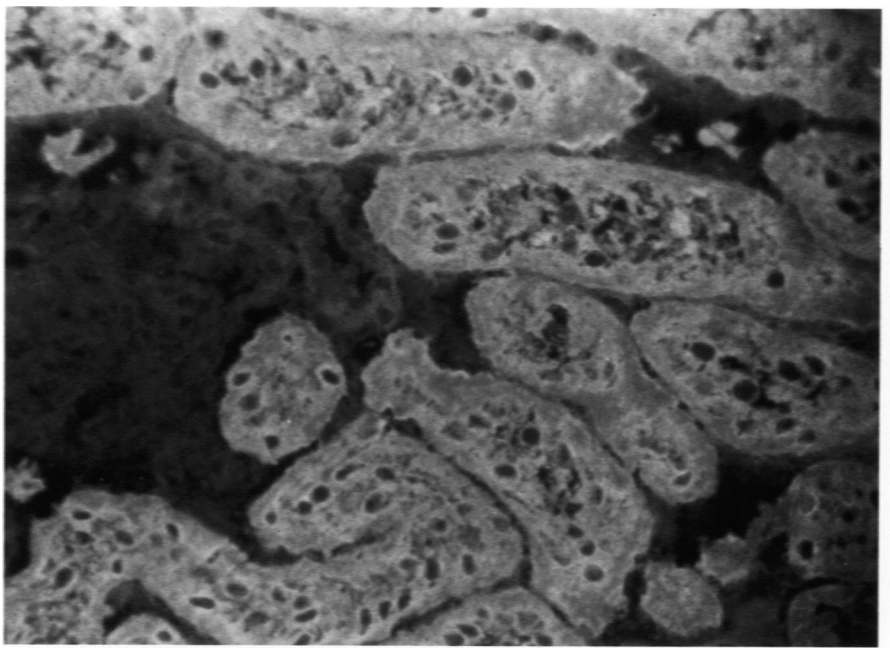

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Fig 11—Section of normal rabbit kidney that was treated with antiserum specific for tubular basement membranes and then stained with fluorescein-labeled antiserum to rabbit IgG. Thin linear staining of basement membranes of proximal convoluted tubules, but not glomerulus or distal convoluted tubules, is seen (× 200). **Fig 12**—Section of normal rabbit kidney that was overlaid with serum from rabbit immunized with kidney and then treated with fluorescein-labeled antibodies to rabbit IgG. Staining of cytoplasm of proximal convoluted tubules, but not the distal convoluted tubules or the glomerulus, can be seen (× 200).

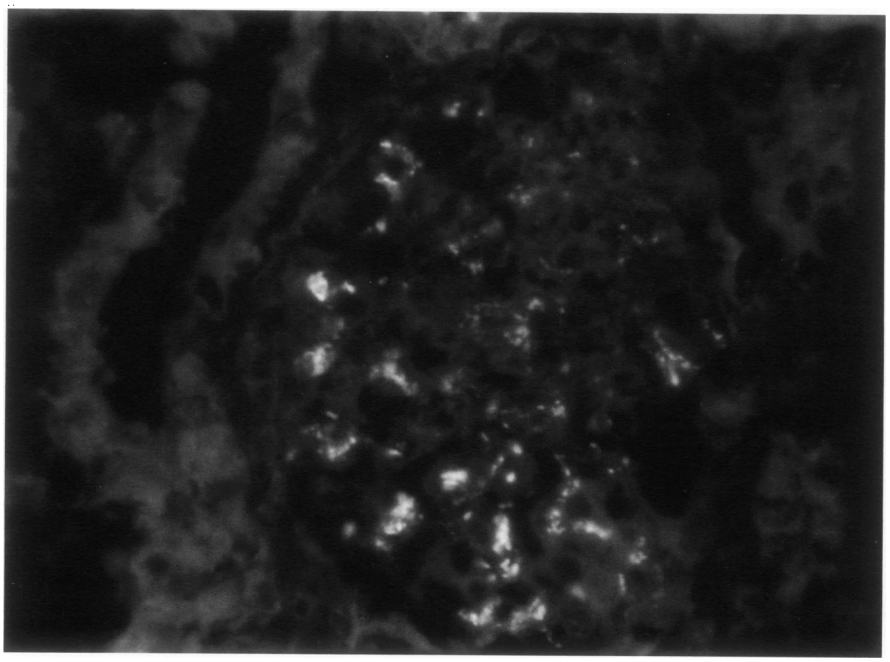

Fig 13—Section of kidney from rabbit 3470 stained with fluorescein-labeled antiserum to rabbit IgA. Granular deposits in mesangial pattern can be seen (× 300).