Demonstration of a Tubular Basement Membrane Antibody in the Pathogenesis of Autoimmune Nephrosis in Rats[1] (37455)

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Heymann and associates (1) in 1959 first described a nephrotic syndrome in rats which had been produced by repeated intraperitoneal injections of complete Freund's adjuvant mixed with a low speed supernate of homogenized rat kidney. Since then this experimental model has been studied extensively and the following conclusions have been drawn regarding its pathogenesis. (a) It is an immunologically mediated glomerulonephropathy in which endogenous (autologous) nonglomerular immune complexes along with $beta_1C$ globulin are deposited along the glomerular capillary wall in a granular pattern on the subepithelial side and produce glomerular injury (2–12). (b) Histologically both by light and electron microscopy the disease has a remarkable resemblance to the chronic membranous glomerulonephropathy in man (10). (c) The nephritogenic antigen responsible for the induction of humoral autoantibody has been identified to originate from the brush border of the epithelial cells or the periluminal portion of the proximal convoluted tubules. It is believed that the antigen normally escapes into circulation from the tubular cells and combines with its autologous humoral antibody to produce pathogenetic antigen–antibody complexes. This brush border antigen (BBAg) has been identified to be a large molecular weight lipoprotein and is implied to be the only nephritogenic antigen in this disease (8, 11).

In the present study evidence is presented that another renal tubular antigen which is localized in the tubular basement membrane (TBMAg) may also be involved in the immunopathogenesis of this disease.

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Materials and Methods. Preparation of antigen. Cold (4°) saline *in vivo* perfused kidneys were obtained from healthy random bred Sprague–Dawley rats. The capsule of the kidney was stripped, renal cortex was separated from the medulla and the cortex chopped with razor blade almost to a thick paste-like consistency. It was gently pushed through a 60-mesh stainless steel wire cloth with the help of a spatula, and the cortical suspension collected in a beaker placed in an ice bath. For each gram of cortical tissue approximately 30–40 ml of normal saline was required to obtain a uniform clotless cortical suspension. It was centrifuged in cold (4°) at 200*g* for 3 min and washed 3 times with cold normal saline to obtain a clear supernatant. The sediment was saved, and the supernatant from the initial centrifugation and subsequent washings were pooled together (supernatant I). Pooled supernatant I was then centrifuged in the cold at 12,000*g* for 20 min, and supernatant II and sediment II saved and lyophilized. The lyophilized material was stored at —20° until used. Protein determinations were done on the lyophilized material (supernatant II and sediment II) by Lowry's method (13).

Production of renal disease. Ten Lewis male rats weighing 150–200 g were injected intraperitoneally every other week with a mixture of complete Freund's adjuvant and supernatant II. The ratio of the volumes of supernatant II and complete Freund's adjuvant were kept the same as previously described by Heyman *et al.* (1). Each rat received 4 mg of protein per injection and a total of 6–10 injections. Urines were examined once a week and total proteinuria determined on a 17-hr urine collection. Protein-

uria in excess of 40 mg/17 hr or 288 mg% was considered significant. Immunizations were discontinued whenever significant proteinuria was noted on two consecutive urine specimens or if significant proteinuria did not develop after 10 injections. Significant proteinuria accompanied by serum cholesterol above 100 mg% and total serum lipids in excess of 400 mg% indicated clinical renal disease with nephrotic syndrome. Methods for quantitation of proteinuria and determination of blood chemistries have been described previously (1).

Immunofluorescent technique. Rats were bled once prior to immunization and every two weeks thereafter prior to each injection after the fourth injection. Serum was separated and frozen at —20° until used.

Fresh normal Sprague–Dawley kidney was quickly frozen in liquid nitrogen and 2–4 μm sections cut in a cryostat. The sections were air dried at room temperature and then reacted with test serum for one hour in a moist chamber at room temperature. They were washed twice with continuous shaking in phosphate-buffered saline pH 7.2 and then reacted with fluorescent-labelled rabbit anti-rat gamma-globulin for another hour in the moist chamber. The sections were again washed twice with continuous shaking in phosphate buffered saline and mounted in 50% buffered glycerol.

The globulin fractions of rabbit anti-rat globulin and rabbit anti-rat $beta_1C$ globulin were conjugated with fluoresceine isothiocyanate as previously described (5). The specificity of the fluorescent conjugate for anti-rat gamma globulin was proven by double diffusion gel precipitation and by immunoelectrophoresis. Immune test sera with positive fluorescence were absorbed with lyophilized supernatant II; 100 mg of lyophilized supernatant II was added to 1 ml of test serum and the mixture allowed to react under continuous shaking for 18 hr at room temperature.

Elution techniques. Gamma-globulins from fresh kidneys of immunized animals were eluted with citrate buffer pH 3.2 as previously described (11).

Results. Results are summarized in Table I. Humoral antibodies were detectable in serum by the indirect immunofluorescent technique in all rats after the 4th injection. Two distinct patterns of staining were seen when the test sera were reacted with normal Sprague–Dawley rat kidney sections. In addition to the bright staining in the periluminal, apical portion of the proximal convoluted tubular cell as previously described (7, 11), a distinct linear staining of the tubular basement membrane was seen in all sera (Fig. 1). Both staining patterns were seen as early as after the fourth injection (7 weeks after immunization) and persisted till the animals were sacrificed (21 weeks from the onset immunization). Appropriate controls including normal rat serum, other tissues such as normal rat liver muscle and lung, and fluoreceinated rabbit anti-rat gamma-globulin alone did not give any significant staining.

When the immune test serum was absorbed with lyophilized supernatant II the two above mentioned patterns of staining were almost completely eliminated. In addition to these two distinct staining patterns, linear staining of the entire Bowman's capsule was consistently seen with all sera; but no staining

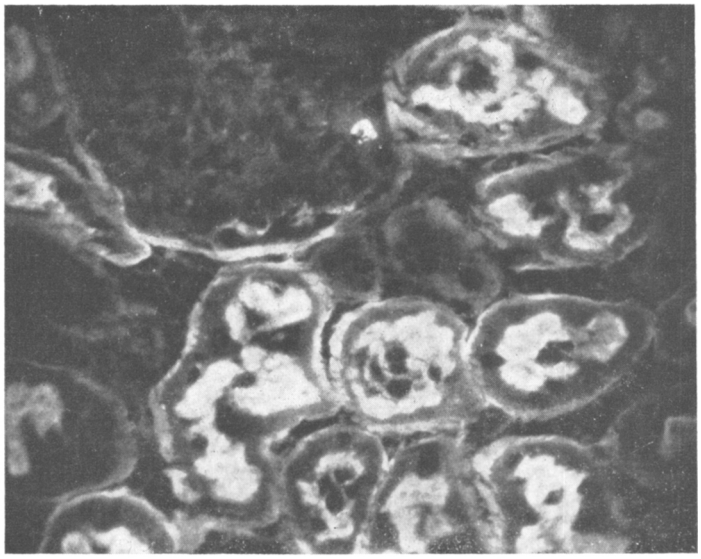
TABLE I. Immunization of Normal Lewis Rats with a Sprague–Dawley Kidney Tubular Fraction and Complete Freund's Adjuvant.

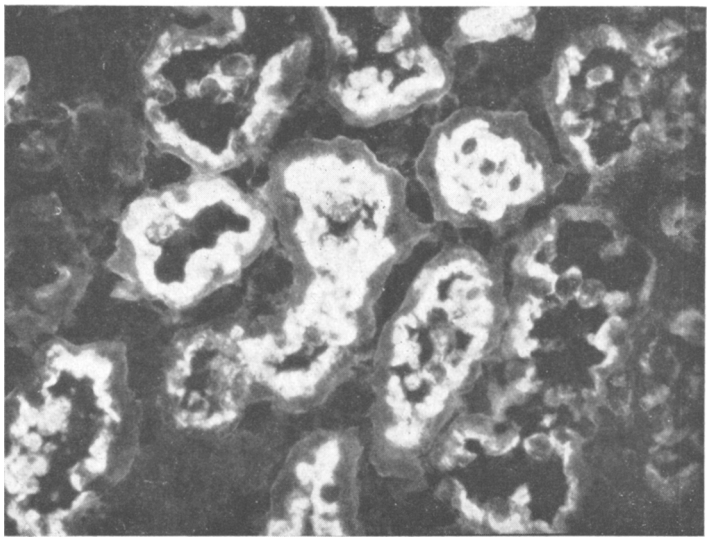
No. of rats	BBAb[a]	TBMAb[b]	Glomerular deposits		Significant proteinuria	Nephrotic syndrome
			Gamma globulin	$Beta_1C$ globulin		
9	9/9	9/9	9/9	9/9	6/9	6/9[c]

[a] Brush border antibody. (Also sometimes referred to as periluminal antibody.)

[b] Tubular Basement Membrane antibody.

[c] 3 rats died spontaneously before the onset of the nephrotic syndrome.





FIGS. 1 and 2. Fluorescent photomicrographs showing two serologic reactions with tubular antigens. Frozen sections from normal Sprague–Dawley rat kidney are stained by indirect immunofluorescence for rat gamma-globulin after reaction with immune serum (Fig. 1) and acid eluate from the diseased kidneys (Fig. 2) of Lewis rat immunized with a mixture of complete Freund's adjuvant and a tubular fraction from normal Sprague–Dawley rat kidney. Two patterns of staining are noticed: (*1*) bright heavy staining in the apical portion and the brush border of the proximal tubular epithelial cells. (*2*) Linear fluorescence along the tubular basement membrane and Bowmans capsule of the glomerulus. No fluorescence in the mesangium or the basement membrane of the glomerulus (upper left corner Fig. 1) is noticed.

was ever detected in the glomerulus either along the capillary loops or in the mesangial area.

Direct immunofluorescent staining for gamma-globulin and $beta_1C$ globulin of the kidneys from the immunized rats showed the typical pattern of granular deposition of host gamma-globulin and $beta_1C$ globulin along

the capillary loops as has been previously reported by several investigators (4–12). No *in vivo* staining for gamma globulin or beta$_1$C globulin was noted along the tubular basement membrane or the periluminal portion of the proximal convoluted tubules in the kidneys of the animals.

The gamma globulin deposits in the glomeruli of the immunized rats were eluted from the kidney cortex at pH 3.2 with citric acid citrate buffer. The eluate, when reacted with sections of normal Sprague–Dawley kidney by indirect immunofluorescent technique, produced the same two patterns of staining as observed with the immune sera, *i.e.*, bright heavy staining along the periluminal apical portion of the proximal convoluted tubule and a fine linear fluorescence along the tubular basement membrane (Fig. 2). Although the linear fluorescence along the tubular basement membrane obtained with the eluate appeared to be somewhat less intense as compared to the one obtained with the immune serum. No fluorescence could be demonstrated when the eluates were reacted with normal Sprague–Dawley rat liver, muscle or lung sections.

Discussion. Heymann's studies (4) first suggested that some autologous nonglomerular antigens may be the nephritogenic antigens in this form of autologous immune complex glomerulonephritis. Edgington (8) *et al.* identified, isolated, and partially characterized the nephritogenic antigen and found it to be a large molecular weight lipoprotein probably a plasma membrane component. Immunohistochemically the antigen (BBAg) was localized in the brush border of the epithelial cells of the proximal convoluted tubules.

Grupe *et al.* (11), subsequently demonstrated autoantibodies to several kidney antigens in the sera of immunized animals but were able to elute only one autoantibody (antibody to BBAg) from the glomeruli of the diseased animals and implied that BBAg was the only nephritogenic antigen.

In the present study, however, we have demonstrated another antigen (TBMAg) which seems to be localized in the tubular basement membrane of the proximal convoluted tubules and may also be nephritogenic. The antibody to this antigen was present and persistent in all immune sera and also could be eluted from the immune deposits in the glomeruli of the diseased animals. Since antibodies to both these antigens (TBMAg and BBAg) were deposited in the glomeruli, the nephritogenicity of TBMAg will only be proved when it is purified.

Since by indirect immunofluorescent technique, antibody to TBMAg is localized in a linear pattern along the tubular basement membrane, it seems that TBMAg is localized in the tubular basement membrane. In view of the above interpretation absence of *in vivo* deposition of tubular basement membrane antibody (TBMAb) along the tubular basement membrane of the kidneys of the immunized animals remains unexplained.

It is of interest to point out that the use of pertussis vaccine as an added adjuvant to complete Freund's adjuvant is not needed for the production of TBMAb and that production of this antibody is not limited to Brown Norway or Lewis/Brown Norway strains of rats only (14).

Summary. Another humoral autoantibody tubular basement membrane antibody (TBMAb) has been demonstrated in the immune sera of Lewis rats in whom autoimmune nephrosis was produced by repeated intraperitoneal injections of a mixture containing complete Freund's adjuvant and a kidney tubular fraction obtained from normal Sprague–Dawley rats. The antibody, by indirect immunofluorescent technique, localized in a linear pattern along the normal tubular basement membrane and Bowman's capsule, but not along the glomerular basement membrane. Also the antibody (TBMAb) could be eluted from the immune deposits in the glomeruli of the diseased animals. These studies suggest the nephritogenicity of this antibody (TBMAb) and point out that at least two antigens (BBAg and TBMAg) may be nephritogenic in this form of experimental autoimmune nephrosis.

Addendum: It has been noted that TBM Ab develops only when Lewis rats are injected with Sprague–Dawley rat kidney and not vice versa.

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