Kidney International, Vol. 31 (1987), pp. 800–807

Identification of a target antigen in human anti-tubular basement membrane nephritis

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Identification of a target antigen in human anti-tubular basement membrane nephritis. Sera from two patients with primary anti-tubular–basement–membrane–mediated tubulointerstitial nephritis, one a renal allograft recipient and the other with spontaneous anti-tubular–basement–membrane disease, were analyzed for the specificity of their autoantibodies. Both sera had circulating antibodies that reacted by ELISA with extracts of tubular basement membrane from several species, but failed to react significantly with extracts of glomerular basement membrane. Reactive antigen was solubilized with 6 M guanidine-HCl, 6 M urea, with reduction and alkylation, and with sodium dodecylsulfate. Digestion of the basement membrane with collagenase released relatively small quantities of antigen from the membrane, and trypsin and pepsin destroyed its antigenicity. The antigenic activity was characterized with respect to its size distribution by gel filtration and by immuno-overlay analysis of protein blots. Collectively, the results indicate that the major reactivity of both sera is directed towards a M_r 58,000 component that is unique to the tubular basement membrane. Minor reactivities toward high molecular weight components common to both glomerular and tubular basement membranes were detected by immuno-overlay analysis. This study identifies an antigen that is involved in human anti-tubular–basement–membrane–mediated tubulointerstitial nephritis, and demonstrates an advantage of the use of denaturing extraction over proteolytic methods to prepare the antigen.

The first animal model of tubulointerstitial nephritis (TIN) induced by antibodies reactive with the basement membrane of proximal tubules (TBM) was developed in 1971 by Steblay and Rudofsky in guinea pigs immunized with rabbit TBM [1]. Immunofluorescence analysis of the kidneys from immunized animals revealed linear deposition of IgG along the TBM but not along the glomerular basement membrane (GBM) or Bowman's capsule. Passive transfer of IgG from immunized animals to naive guinea pigs induced TIN comparable to that seen in immunized animals [2–4]. Passive transfer of lymph node cells from immunized animals to normal animals failed to elicit tubular damage [3]. Bovine TBM administered to guinea pigs in conjunction with cyclophosphamide led to the conclusion that anti-TBM antibodies were the mediating factor of TIN in this model [5]. A similar model was developed in Brown/Norway or Lewis X Brown/Norway F1 hybrid rats immunized with bovine TBM [6].

GBM has also been used as an immunogen to induce nephritides in animals. In one study, immunization with GBM resulted in tubular damage followed, 14 days later, by glomerular damage [7]. Other studies demonstrated that immunization with heterologous GBM led to the production of antibodies directed to GBM and TBM, whereas immunization with TBM produced antibodies against TBM only [8–11]. Conclusions drawn from these studies were that GBM was eliciting responses to common domains of type IV procollagen and TBM immunization was resulting in responses to noncollagenous glycopeptides present in TBM only.

Several studies have been performed on the isolation and analysis of nephritogenic antigens from TBM. Graindorge and Mahieu [12] digested human TBM with collagenase, and isolated a M_r 70,000 protein from the solubilized material. Its amino acid and carbohydrate composition was characterized, and it was used to establish a radioimmunologic assay for TBM antigen. In that assay, nine of 11 patients with glomerulonephritis, where linear staining of both GBM and TBM was observed, had detectable antibodies toward the TBM antigen. Furthermore, sera from two patients with TBM antibodies that appeared secondarily to an immune–complex glomerular disease associated with a nephrotic syndrome demonstrated reactivity toward the purified protein. Wakashin et al [11] isolated a trypsin solubilized protein (M_r 30,000) from human TBM, and used it as an immunogen to generate autoimmune renal disease in goats and mice. The TBM of goat, guinea pig, mouse and man all reacted with the antibodies toward the human TBM antigen by indirect immunofluorescence indicating a shared specificity among species. Zanetti and Wilson identified a molecule precipitated by TBM antibodies from Brown/Norway rats immunized with bovine TBM [13]. This molecule, obtained from TBM by collagenase digestion, had a molecular weight of 42,000 by SDS-PAGE. The antigen was present in the Brown/Norway, but not in the nonsusceptible Lewis strain and was proposed to be important in triggering autoantibody formation in this model. A TBM antigen recently isolated by Clayman et al [14] is a noncollagenous glycoprotein of M_r 48,000. The protein was prepared by collagenase digestion of the TBM and was purified by immunoabsorption using a purified monoclonal antibody. It contains low levels of hydroxyproline, suggesting

Received for publication June 3, 1985,
and in revised form May 27, 1986

that it could be derived from a collagenous precursor. This peptide induced TBM-specific antibody formation and renal–interstitial cell infiltrates in guinea pigs.

The pathogenesis of TIN is, in the majority of patients, poorly understood. In a small number of patients the disease is associated with anti-TBM antibodies. Immunofluorescence microscopy studies of their kidneys reveal the presence of linear deposits of IgG and complement along the basement membrane of the tubules. Evidence that these linear deposits are the consequence of binding in vivo of specific antibodies may come from the demonstration of anti-TBM antibodies in the serum or kidney eluates. Anti-TBM antibodies have been reported in conjunction with anti-glomerular–basement–membrane antibody–mediated glomerulonephritis and with immune complex glomerulonephritis, in patients treated with certain drugs, or following kidney transplantation [15]. The observation of anti-TBM antibodies as an isolated phenomenon in patients with TIN is distinctly rare [16]. Evidence for the pathogenicity of anti-TBM antibodies in man is obviously difficult to obtain [17]. Final proof can only be provided by experiments aimed at inducing TIN in suitable laboratory animals by passive transfer of human anti-TBM antibodies.

The identification and characterization of TBM antigens involved in human TIN is essential to gain further understanding of the disease process and to correlate with animal models of the disease. The present study was undertaken in order to identify TBM antigen(s) reactive with antibodies from patients with a pure form of TIN. The results indicate that a protein of M_r = 58,000 binds antibodies in the sera from two patients with a primary anti-TBM-induced TIN. This protein can be extracted from TBM in denaturing solvents; thus procedures leading to its purification can be developed while avoiding potential problems associated with enzymatic solubilization.

Methods

Human anti-TBM antibodies

Serum used in the present studies was obtained from two patients. The first patient (TIN-1) was a 27-year–old female, admitted to the Buffalo General Hospital (Buffalo, New York, USA) in 1983 because of acute renal failure. Her kidney biopsy showed TIN with infiltration of the interstitium with mononuclear as well as neutrophilic polymorphonuclear leukocytes. By immunofluorescence microscopy, linear deposits of IgG and C3 were present along the basement membrane of the proximal tubules. Immune reactants were not found in the glomeruli. The second patient (TIN-2) was a 36-year–old male with end–stage kidney disease of unknown origin who received a kidney allograft in August of 1983. Two months later he was admitted to the Buffalo General Hospital on suspicion of acute kidney transplant rejection. The biopsy revealed TIN characterized by interstitial infiltration of mononuclear cells and a few neutrophilic polymorphonuclear leukocytes. Immunofluorescence microscopy studies demonstrated linear deposits of IgG and C3 along the basement membrane of the proximal tubule. Immune deposits were absent from the glomeruli.

The serum of both patients contained antibodies that reacted in vitro exclusively with the basement membrane of proximal tubules of human as well as animal (rabbit, guinea pig, rat, mouse) kidneys. Differences in reactivity between the sera of the two patients could not be detected.

Basement membrane

TBM and GBM from human, bovine, Brown/Norway rat, and rabbit sources were prepared as follows. Human glomeruli and tubules were prepared by the method of Langeveld et al [18]. This method involves forcing cortical kidney tissue through a 500 μm stainless steel sieve and washing it with 0.15 M NaCl which was 10 mM in EDTA, 5 mM in epsilon–amino caproic acid, 5 mM in N-ethylmaleimide and 1 mM diisopropylfluorophosphate through a series of successively finer sieves. The glomeruli were trapped on 125 and 106 μm sieves. The tubules were captured on 45 and 38 μm sieves. The products were sonicated in 1.0 M NaCl with the above inhibitors included. The resulting basement membrane was washed three times with the 1.0 M NaCl solution, three times with water, then frozen and lyophilized. Rat TBM was prepared by the method previously described [19]; and rabbit TBM was prepared by the method described [20] using a polytron tissue disruptor instead of forcing tissue through the coarse sieve.

Antigen solubilization

Basement membrane was extracted overnight in 6 M guanidine-HCl/0.05 M Tris, pH 7.5 at 37°C in the presence of the protease inhibitors listed above, excluding the diisopropylfluorophosphate. Other extractions were done at the same temperature, but using either 6 M urea, 0.05 M Tris-HCl pH 7.5 or 8 M urea, 0.1 M Tris-HCl, pH 7.5 in the presence of protease inhibitors. In some cases the protein was ethanol precipitated by adding a tenfold aliquot of alcohol at 0°C followed by centrifugation. For gel filtration analysis, an 8 M urea, 0.1 M Tris, pH 7.5 extract was reduced in volume on an Amicon system using a YM-10 filter and applied to a Sepharose CL-4B column (2.5 × 90 cm) equilibrated in 6 M urea, 0.1 M sodium phosphate, pH 7.4. The effluent from the concentration step and from the gel filtration column was monitored for absorption at 230 nm and for reactivity by ELISA.

Collagenase. Bacterial collagenase (Worthington Biochemical Corp, Freehold, New Jersey, USA, CLSPA, 540 units/mg or Advanced Biofactures Corp., 330 μ/mg) was dissolved in 50 mM HEPES buffer containing 10 mM $CaCl_2$, 4 mM N-ethylmaleimide, 5 mM benzamidine-HCl, 25 mM epsilon–amino caproic acid, pH 7.5. Human and rabbit TBM were incubated with 70 μ collagenase per mg TBM with stirring at 37°C overnight. The final concentration of TBM was adjusted to 8 mg/ml by the addition of HEPES buffer with protease inhibitors.

Pepsin. Porcine stomach mucosal pepsin (Sigma Chemical Co., St. Louis, Missouri, USA, 3200 units/mg) was dissolved in 0.5 N acetic acid to a concentration of 1 mg/ml. The enzyme was added to human TBM (4 mg/ml in 0.5 N acetic acid) at an E/S ratio of 10% (wet/wt), and incubated with stirring overnight at 4°C.

Trypsin. Trypsin (Worthington, TPCK, 236 units/mg) was dissolved in 0.001 N HCl at concentration of 1 mg/ml. TBM was suspended in 0.5 M Tris-HCl, pH 7.6 and trypsin solution was added to give an E/S ratio of 1% (wet/wt). Digestion was carried out at 37°C overnight with stirring.

To prepare the samples for gel electrophoresis, aliquots of the digestion mixtures were precipitated at 4°C with a tenfold

excess of ethanol. This was centrifuged, and the pellets dissolved in gel–electrophoresis sample preparation buffer.

Reduction and alkylation. Human TBM was reduced by the addition of beta-mercaptoethanol (1 mmol per 3 to 4 mg TBM) in 6 M guanidine-HCl, 0.05 M Tris-HCl, pH 8.5 in a boiling water bath for 2 to 3 minutes. Following this, iodacetamide (2 mmol) was added with stirring. The suspension was then clarified by centrifugation, filtered, precipitated and prepared for electrophoresis analysis.

Electrophoresis analysis

Polyacrylamide gel electrophoresis was done in discontinuous buffers in SDS using the procedures as described by Laemmli [21].

Immunochemical methods

ELISA was done as previously described [22–24]. Antigen was coated to a polystyrene microtiter plate in 0.2 M sodium carbonate buffer, pH 9.5, overnight at ambient temperature. Serum was diluted in 0.05 M sodium/potassium phosphate, pH 7.5, 0.15 M NaCl, 0.05% Tween 20, 0.2% bovine serum albumin and incubated on the coated plates for one hour. Plates were rinsed with 0.15 M NaCl, 0.05% Tween 20, incubated with anti-human IgG-alkaline phosphatase conjugate for an hour, then tested for enzyme activity using p-nitrophenyl phosphate as substrate. Enzyme activity was measured by monitoring absorbance at 405 nm. Assays were performed in triplicate. Competitive ELISA was attempted by mixing equal volumes of diluted antibody and antigen in the phosphate buffer, followed by incubation at 4°C overnight. The incubate was then transferred onto coated microtiter plates, and incubated for one hour. Plates were rinsed and incubated with conjugate and substrate as above.

Immunoblot (Western blot) was carried out by transferring electrophoresed material from PAGE onto 0.45 μm nitrocellulose paper [25]. Following transfer, the paper was blocked by incubation in 0.2 M sodium carbonate, pH 9.5, containing 2% bovine serum albumin for a minimum of one–half hour. Test serum was diluted as above and incubated for a minimum of one hour with the blocked nitrocellulose paper. The nitrocellulose paper was rinsed and incubated with anti-human IgG-horseradish peroxidase conjugate for a minimum of one hour, then washed and enzyme activity was tested with H_2O_2-diaminobenzidine substrate. In some cases, blots were stained for protein using India ink [26].

Molecular weight determination

Molecular weight measurements were made from protein blots of electrophoresis gels. Samples and molecular weight standards were run in duplicate and blotted onto nitrocellulose paper. The paper was then cut, and one half was stained for protein with India ink [26], and the other half was analyzed for reactivity with TIN sera by overlay assays. Molecular weights of reactive bands were determined by correlating the position of bands detected by overlay assay with the duplicate blot stained for protein. Molecular weight standards included bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate and carbonic anhydrase.

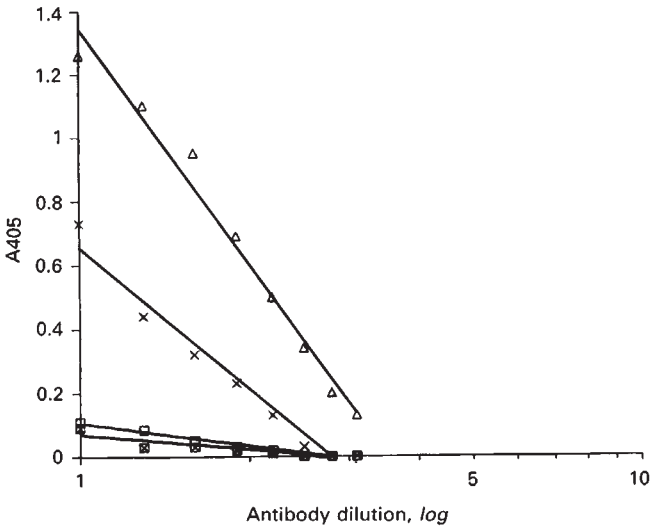

Fig. 1. *Binding of serum antibodies from patients with tubulointerstitial nephritis to tubular basement membrane.* Human TBM was extracted with 6 M guanidine HCl, 0.05 M Tris, pH 7.5, at 37°C overnight. The solubilized protein was diluted 1:10 in carbonate buffer, pH 9.0, and coated onto a microtiter plate using 20 μg protein per well. The reactivity of anti-TBM sera (top two curves) with the TBM extract is compared by ELISA with normal human serum (bottom two curves) at dilutions from 1:10 to 1:1024. Assays were performed in triplicate, and the results represent the averages.

Results

Reaction of TBM extracts with TIN sera

Human TBM was extracted at 37°C in 6 M guanidine buffer and in 6 or 8 M urea buffers in the presence of protease inhibitors. Antigenic activity solubilized by guanidine as determined by ELISA is demonstrated by the antibody titration curves presented in Figure 1. Antigen titration (not shown) showed diminished reactivity with increasing dilution of the antigen. Reactivity of the antisera with extracts of GBM was not significantly above background. The results demonstrate that the autoantibody from the TIN patients bind an antigen present in guanidine extracts of TBM. Both sera have readily detectable levels of TBM antibodies, whereas control sera are consistently negative. When guanidine–insoluble TBM was subsequently solubilized by reduction of disulfide bonds, only traces of additional activity were detected. Reduction of disulfide bonds in the guanidine soluble fraction did not alter its reactivity with human TBM antibodies. This result demonstrates that guanidine releases essentially all the antigen from TBM and that its reactivity with the human TBM antibodies is not altered by reduction. Urea extraction also was effective in solubilizing the antigenic activity from TBM; however, about 15 to 25% remained in the insoluble matrix, as determined by ELISA on guanidine extracts of urea–insoluble TBM and by Western blot analysis performed on the various fractions.

Efforts were made to quantitate the extracted antigen in a competitive ELISA. Extracts were diluted in 0.05 M phosphate, pH 7.5, 0.15 M NaCl, 0.05% Tween-20, 0.2% bovine serum albumin, then incubated with TIN sera. No competition was realized, hence the antigen either failed to bind in solution and/or dilution of the denaturant caused loss of reactivity.

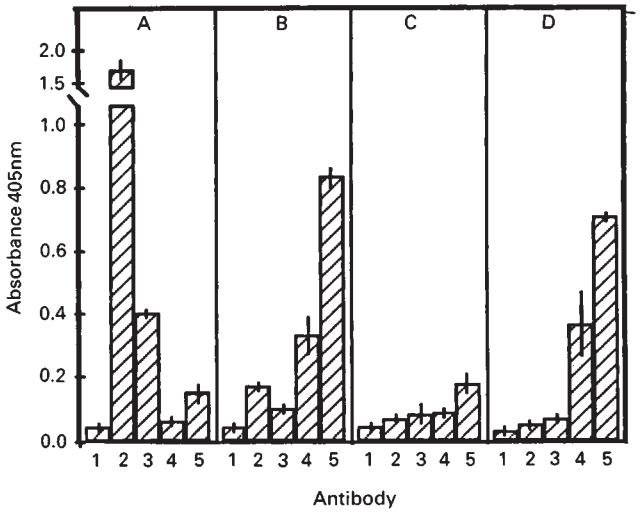

Fig. 2. *Comparison of TBM antibodies, Goodpasture antibodies and 7S collagen antibodies for specificity toward TBM and GBM fractions.* ELISAs were performed on guanidine–solubilized TBM (**A**) and GBM (**C**); and on the guanidine insoluble residue following digestion with bacterial collagenase (**B** and **D**). B represents ELISAs performed on guanidine–insoluble TBM digested with collagenase and D represents guanidine–insoluble GBM digested with collagenase. The test sera represented by lanes 1–5 in each panel (A–D) were as follows: lane 1, normal human serum diluted 1:20, lane 2, TIN-1 serum at 1:100; lane 3, TIN-2 serum to 1:50, lane 4, Goodpasture serum at 1:50; and lane 5, rabbit antiserum to 7S collagen at 1:500. ELISA measurements were made in triplicate as described in Figure 1, using 10 μg of coating antigen per well. Values represent the mean ± SD.

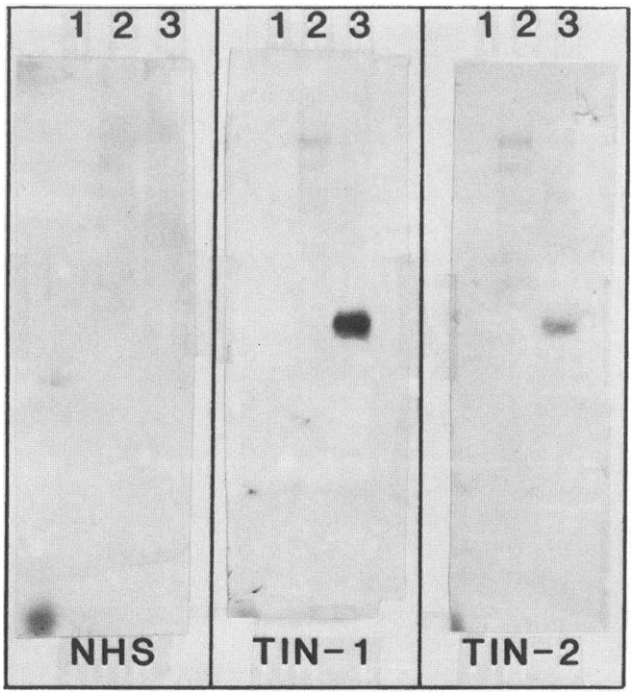

Fig. 3. *Western blot analysis demonstrating the presence of an antigen in TBM that is reactive with human TBM antibodies.* Lanes 1–3 in each panel represent: 1) molecular weight standard protein mixture to demonstrate the absence of nonspecific binding; 2) guanidine–extracted human GBM and 3) guanidine–extracted human TBM. Samples of 2.5 μg extracted protein and 5 μg of each protein in the standard mixture were applied to an electrophoresis gel. Following electrophoresis, samples were blotted by electrophoretic transfer onto nitrocellulose sheets. The nitrocellulose paper was then cut for incubation in normal human serum (NHS); and with the human TBM antisera (TIN-1 and TIN-2) diluted 50×. The blots were developed with anti-human IgG-horseradish peroxidase conjugate and H_2O_2-diaminobenzidine.

ELISAs designed to demonstrate binding and nonbinding of antibodies in TIN sera to various basement membrane preparations are shown in Figure 2. These results indicate that both TIN sera react with guanidine · HCl extracts of human TBM (2A, lanes 2 and 3) but not with extracts of GBM (2C, lanes 2 and 3). Binding of antibody to guanidine extracts of human GBM was not significantly greater than incubations with normal human serum. Reactivity of TIN sera with collagenase digestion products of guanidine–insoluble basement membranes was not significantly greater than that of normal human serum and therefore may be considered to be negative. This result is consistent with the observation that guanidine solubilizes nearly all of the antigenic activity from the TBM, hence digestion of the guanidine insoluble fraction does not release additional reactivity. Collagenase digestion of whole TBM, that is, not previously extracted with guanidine, released only about 5 to 10% of the potential reactivity. Collectively, these results are suggestive of a noncollagenous derivation of the TBM antigen(s). Both the Goodpasture antigen and 7S collagen are released from guanidine insoluble TBM upon collagenase digestion (Figure 2B) indicating that Goodpasture antibodies and antibodies toward 7S collagen are distinct from the TBM antibodies.

Identification of a reactive TBM antigen by western blotting

Immuno-overlay analysis was used to further characterize the antigen recognized by the TIN patient's sera. Both sera showed a strong reaction with a M_r about 58,000 band in SDS-soluble human TBM, whereas GBM was negative for this component, as shown in Figure 3. Three faintly staining bands of M_r 100,000; 150,000 and 200,000 were also detected with the two anti-TBM sera, but not with control sera. These bands were seen in extracts of both TBM and GBM (Figs. 3 and 4B). The latter result suggests that presence of antibodies specific for components common to both TBM and GBM or nonspecific binding to common components. Analysis of blots of antigen extracted with either 6 M guanidine or 6 M urea in 0.05 M Tris, pH 7.5 gave similar results. An intensely staining, M_r 58,000 antigen was always detected, and the three faintly staining bands were generally observed. Figure 4A presents a blot of guanidine solubilized human TBM and GBM (lanes 2 and 3) stained for protein with India ink. Multiple protein bands spanning a wide molecular weight range are detected with the ink stain. The specificity of one anti-TBM serum for components present in the guanidine extract is shown is 4B, lanes 1 and 2. The intensely staining band present in lane 2 is unique to TBM, whereas the three very lightly staining bands (lanes 1 and 2) in the upper 1/3 of the gel profile are present in both the GBM and TBM extracts. To determine whether disulfide bonds are important to the integrity of the TBM antigen, human TBM was reduced and alkylated, and analyzed by Western blotting (not shown). The reduced TBM sample retained its reactivity with the anti-TBM sera, and there was no change in its apparent molecular weight.

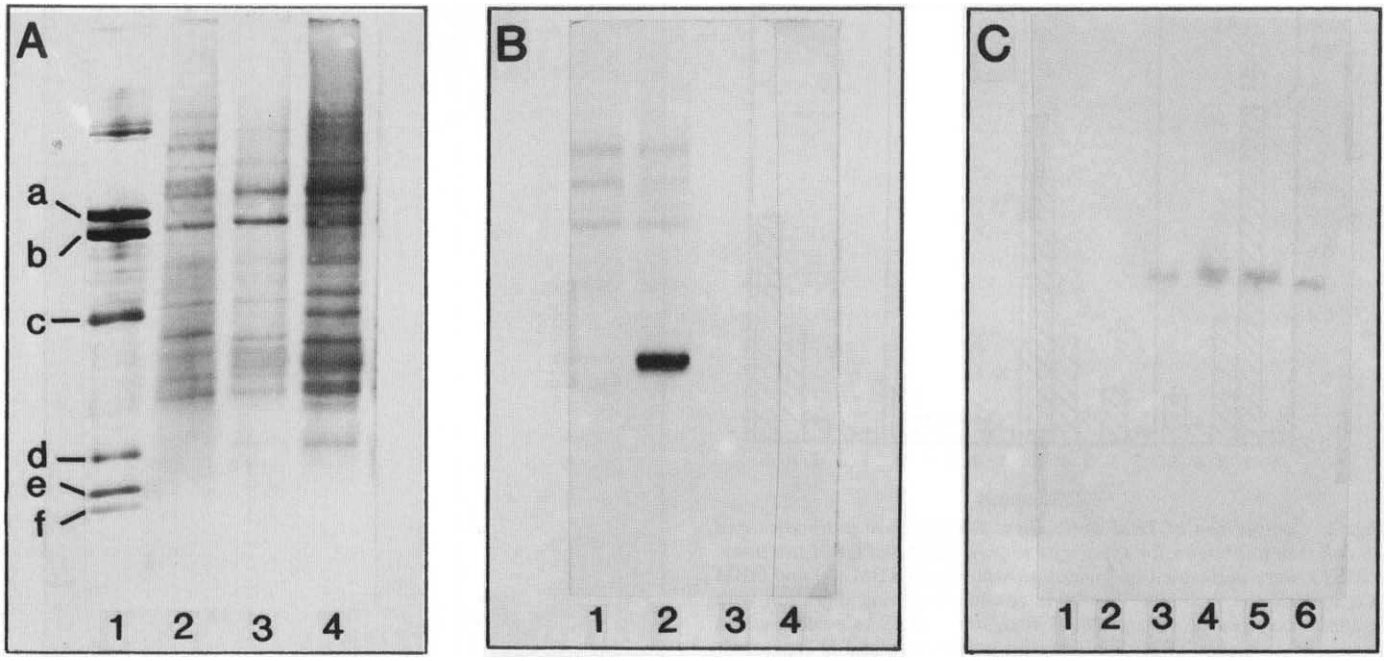

Fig. 4. *Comparison of the reactivity of human TBM antibodies with extracted, and enzyme solubilized TBM; and comparison of reactivity among species.* **A.** Electrophoresis of 6M guanidine-HCl soluble human GBM (lane 2), human TBM (lane 3) and rabbit TBM (lane 4). Total protein was stained with India ink. Lane 1 represents molecular weight standards consisting of: a) collagen I, alpha 1 chain; b) collagen I, alpha 2 chain; c) bovine albumin, M_r 66,000; d) egg albumin, M_r 45,000; e) glyceraldehyde-3-phosphage dehydrogenase, M_r 36,000; and f) carbonic anhydase, M_r 29,000. **B.** Immuno-overlay analysis of protein blots of guanidine-HCl soluble human GBM and TBM (lanes 1 and 2), collagenase digested TBM (lane 3) and trypsin digested TBM (lane 4). Samples of 5 μg protein were applied to each lane in A and B. The overlay assay was done using the high titer serum at 50×. **C.** Immuno-overlay analysis of: (1) molecular weight standard mixture, (2) SDS-solubilized human GBM; (3) human TBM; (4) rabbit TBM; (5) rat TBM and (6) bovine TBM. Samples consisting of 2.5 μg of SDS-soluble human GBM and TBM and 6.0 μg each of SDS-soluble rabbit, rat and bovine were applied to the gel represented in **C.**

Tubular basement membrane antigens in other species

In order to test for the presence of reactive TBM antigens in nonhuman species, extracts of human, rabbit, rat and bovine TBM were compared by ELISA and by immuno-overlay with the highest titer anti-TBM serum. ELISA of guanidine extracts demonstrated reactivity in the TBM from each of these species. A complex distribution of protein constituents in the extracts was seen for each species, as determined by SDS gel electrophoresis. The complexity of the extracted protein mixture from human and rabbit TBM is shown in the ink stained protein blot in Figure 4A. By contrast, the immuno-overlay analysis showed a single major reactive band in the material extracted from the TBM of each species examined (Fig. 4C). The reactive band from human, rabbit and rat TBM had an apparent molecular weight of 58,000; whereas the bovine antigen consistently gave an apparent molecular weight of 56,000. Three lightly–staining bands in the molecular weight range of 100,000 to 200,000 were evident in some extracts of human GBM and TBM, and rabbit TBM.

Protease sensitivity and partial purification

In an effort to find reactive protein fragments which might correspond to the nephritogenic antigens identified by others [11–14], whole human TBM (that is, non-extracted) was digested with collagenase, trypsin, and pepsin. Following digestion by these proteases, the reaction products were electrophoresed, transblotted, and analyzed for protein and for reactivity with anti-TBM sera. Although protein bands were evident in the ink stained blots and in silver stained gels run simultaneously, there was no binding of antibodies to the transblotted material from trypsin and pepsin digests, and only traces of a M_r 58,000 band were seen in collagenase digests (Fig. 4B lanes 3 and 4; pepsin digest not shown). This suggests that the antigen is either destroyed by these proteases, or it is not released from the TBM by these methods. To test the latter possibility, the insoluble pellet remaining after digestion with the various enzymes was extracted with 6 M urea and analyzed by immuno-overlay. The extracts of the pellet remaining after trypsin and pepsin digestion of TBM did not contain any reactive polypeptides. The extracted pellet remaining following collagenase digestion contained a reactive polypeptide having of the same mobility as the controls (not shown). By ELISA and visual examination of the Western blots, the amount of antigen present in the collagenase digestion pellet was the same as in the controls. Thus it appears that collagenase is ineffective in releasing significant amounts of the antigen from TBM whereas trypsin and pepsin destroy it.

Immuno-overlay experiments suggest that the M_r 58,000 TBM component is the component primarily responsible for the anti-TBM reactivity of the two human sera. It was important to establish additional evidence for this, and to rule out the possibility that reactivity might exist that would ordinarily

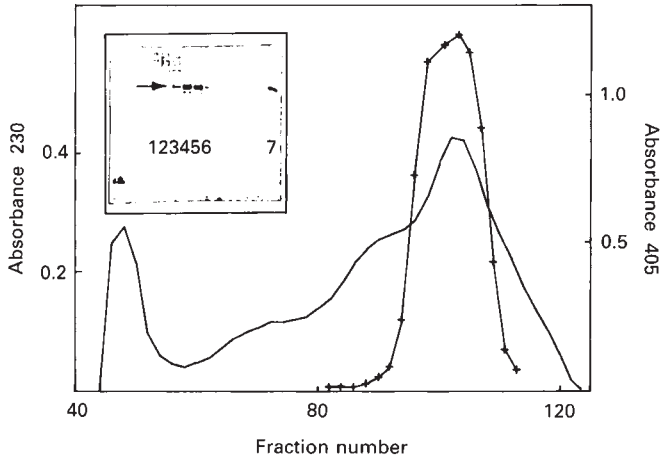

Fig. 5. *Partial fractionation by gel filtration of protein reactive with human anti-TBM sera.* Human TBM was extracted with 8 M urea, 0.1 M Tris-HCl, pH 8.5, and solubilized protein was concentrated and applied to a Sepharose CL-4B column (4 × 90 cm). Column eluate was monitored by absorbance at 230 nm (—) and for binding of antibodies from the high titer serum (+—+) by ELISA. ELISA was performed in triplicate and the average values are represented. The inset represents column fractions that are reactive on immuno-overlay analysis. Lanes 1–6 represent column fractions 84, 90, 96, 100, 104, and 110, respectively. The major reactive, M_r 58,000, component is indicated by the arrow. Its appearance in the immuno-overlay coincides with the major ELISA reactivity. The elution of high molecular components that are reactive by immuno-overlay coincides with very low ELISA reactivities (lanes 1 and 2, representing fractions 84 and 90). Lane 7 represents a sample of the starting material applied to the column.

escape detection by the immuno-overlay techniques, that is, extremely large components would not penetrate a polyacrylamide gel, and extremely small ones would likely be lost during blotting of electrophoresis gels. For this purpose, an 8 M urea, 0.1 M Tris HCl, pH 7.5 extract of human TBM was subjected to gel filtration analysis on a column of Sepharose CL-4B. Fractions eluted from the gel filtration column were monitored for protein and for reactivity toward the high titer anti-TBM serum, as shown in Figure 5. All of the ELISA reactivity was found in the region where proteins of M_r 50,000 to 100,000 were eluted. The reactivity was thus confined to the size range of reactivities observed by immuno-overlay. Fractions from the gel filtration column were then electrophoresed, and the presence of the M_r 58,000 reactive component was confirmed by immuno-overlay (Fig. 5, insert lanes 3–6). The faintly staining higher molecular weight components were also detected by this technique in the gel filtration fractions (Fig. 5, inset lanes 1–3). The high molecular weight components present in lanes 1 and 2 represent gel filtration fractions 80 and 85, where no ELISA reactivity was detected, suggesting that they may not react by ELISA. On the basis of the present study we can conclude that the M_r 58,000 component accounts for nearly all of the reactivity with the human anti-TBM sera, and that traces of reactivity with several high molecular weight components ranging from 100,000 to 200,000 are also present. The M_r 58,000 reactive component is unique to TBM whereas the trace reactive components are present in both TBM and GBM.

Discussion

This study identifies a M_r 58,000 TBM component that is bound by autoantibodies from two patients with anti-TBM mediated TIN. The M_r 58,000 antigen accounts for nearly all of the reactivity that can be detected by immuno-overlay analysis of protein blots. ELISA of TBM extracts resolved by gel filtration demonstrates that nearly all reactivity is present in fractions in the molecular weight range of 50,000 to 100,000, further supporting the conclusion that the M_r 58,000 component is the nephritogenic antigen. Traces of reactivity toward TBM and GBM components of M_r 100,000, 150,000 and 200,000 were detected by immuno-overlay analysis. As monitored by ELISA, the reactivity of the latter components was not significantly above background. The evidence for this comes from analysis of GBM extracts which contain the reactive components by immuno-overlay analysis (Fig. 4B) but are not reactive by ELISA (Fig. 2). In addition, gel filtration fractions from TBM extracts that contain only high molecular reactivities by immuno-overlay analysis do not demonstrate significant reactivity by ELISA. In view of their low reactivities and since they are not TBM specific, it seems unlikely that these components are important in the disease mechanism. This study does not rule out the possible existence of antigens that do not react in the immuno-overlay assay, but the present evidence suggests that this is unlikely.

Reduction of its disulfide bonds did not change the electrophoretic mobility of the antigen, hence the protein probably consists of a single polypeptide chain, and disulfide bonds are not necessarily for its antigenicity. Reduction of the insoluble residue following guanidine or urea extraction of TBM failed to reveal any additional antigenically reactive proteins, although this procedure solubilizes greater than 95% of the basement membrane. Thus guanidine or urea extractions are sufficient to solubilize all of the antigen of interest.

Several reports have described TBM antigens involved in experimental and human TIN [11–14]. The relationship among the various antigens is not known at the present time. The dissimilarity in their molecular weights (M_r 58,000 in this study versus 70,000; 48,000 and 42,000 for collagenase solubilized antigens and 30,000 for a trypsin solubilized antigen) is suggestive of either multiple antigens or that various partial degradation products of a common antigen have been characterized. A common feature of the more recently characterized antigens [13, 14] is that they are apparently absent in certain animal strains such as the Lewis rat. Indeed, the antibodies characterized in this study failed to bind Lewis rat kidney as analyzed by indirect immunofluorescence microscopy. Furthermore, the antigen has been identified in bovine, rabbit, guinea pig, rat, mouse, as well as human kidneys by the combination of indirect immunofluorescence analysis and immuno-overlay analysis. This result suggests that the antigen identified in this study is a highly conserved molecule that may correspond to the ones that have recently been studied by Zanetti and Wilson [13] and Clayman et al [14] in experimental models of TIN. Graindorge and Mahieu prepared and characterized the first TBM antigen recognized by antibodies from cases of human TIN [12]. Their M_r 70,000 antigen was bound by antibodies from two patients

with TIN secondary to immune complex glomerulonephritis, and it was also bound by antibodies from several cases of glomerulonephritis that demonstrated linear GBM and TBM staining and from cases of lupus nephritis. Like the antigen identified in this study, their antigen is TBM specific. Although the observed apparent molecular weight differs between their antigen and the one identified here, it seems probable that we are dealing with the same molecule. A seemingly less plausible alternative is that two TBM specific antigens exist, one of which is bound by antibodies form cases of primary TIN and another by antibodies from cases of secondary TIN and other renal diseases. Thus, the various antigens that have been identified display features in common, but differences also exist, especially with respect to their molecular weights. In order to unequivocally establish the relationships among the various antigens, it will be necessary to purify at least one of them and demonstrate their binding by antibodies in the various models and human disease, or to establish the chemical identity (or nonidentity) between the various purified antigens.

The antigens isolated by Graindorge and Mahieu [12] and Clayman et al [14] are glycoproteins containing low levels of 4-hydroxyproline. The presence of 4-hydroxyproline in their antigens, and the fact that they are solubilized by collagenase suggests that they could be noncollagenous domains of collagenous molecules. By analogy, the Goodpasture antigen, as solubilized by collagenase digestion, contains low levels of 4-hydroxyproline [25]. The Goodpasture antigen has been localized to the globular domain of collagen IV of GBM [27].

The extracts containing our antigen contain 4-hydroxyproline [20] which would be consistent with a possible collagenous origin for our extracted antigen; however, since the extracts contain many other components, meaningful conclusions cannot be made with respect to the composition of our antigen at this point. On the other hand, evidence for a noncollagenous origin for our antigen must be considered. While collagenase releases some antigen, very little of its is solubilized by collagenase compared with the extraction procedure. That which is solubilized by collagenase is of the same molecular weight by SDS gel electrophoresis as extracted antigen, suggesting that the effect of collagenase may be indirect, that is, collagenase may effect solution of the antigen by degrading a collagenous molecule to which the antigen is bound. Indeed, the presence of 4-hydroxyproline in the antigen preparation of Clayman et al [14] could be due to the presence of collagenous molecules that are associated with the antigen, and are not removed during purification by immunoabsorption chromatography. Likewise, the antigen prepared by Graindorge and Mahieu may be contaminated by other components of similar electrophoretic mobility, since it was prepared by preparative gel electrophoresis.

Knowledge of the biochemical properties of this antigen is prerequisite to an understanding of its function in the normal kidney and its role in disease. It will thus be important to develop methods to isolate the antigen in quantities sufficient to perform detailed biochemical analyses. By use of the extraction method of solubilization, it should be possible to obtain sufficient quantities of antigen to permit its purification and analysis, while avoiding the potential complications of the proteolytic solubilization methods.

Acknowledgments

This research was supported by NIH grants AM 26178 and AM 36906. The technical assistance of Ms. Anjana De and Ms. Parvin Todd, and the secretarial assistance of Ms. Jan Wojcik is appreciated.

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