ISOLATION AND CHARACTERIZATION OF THE NEPHRITOCGENIC ANTIGEN PRODUCING ANTI-TUBULAR BASEMENT MEMBRANE DISEASE

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The use of purified self-antigens can facilitate the further analysis of pathogenic mechanisms in autoimmunity (1, 2). This report describes the isolation of the nephritogenic antigen of anti-tubular basement membrane (α-TBM)–induced interstitial nephritis. First described by Steblay and Rudofsky (3) over a decade ago, this experimental lesion is now induced in a variety of inbred strains of mice, rats, and guinea pigs after an injection of rabbit renal tubular basement membrane (renal tubular antigen; RTA) in complete Freund’s adjuvant (CFA). Disease-susceptible rodents develop α-TBM antibodies after 9–10 d, and an intense interstitial infiltrate by 2–7 wk, depending on the species (3-5). Phenotypic characterization of the predominantly mononuclear cell infiltrate reveals the presence of an admixture of T cells, B cells, macrophages, and natural killer cells (6, 7). Additional studies have provided insights into the immunoregulation (8, 9), effector mechanisms (10, 11), and immunogenetics of disease expression (12, 13). Regarding the last of these, several groups have analyzed susceptibility to α-TBM disease in different rat strains (5, 14-16). Certain strains (LEW, MAXX, and W/F, for example) do not develop disease, in part, because they lack the appropriate nephritogenic tubular antigen expressed by the prototypically susceptible BN rat (5, 12, 14). This difference among rat strains can be

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Abbreviations used in this paper: α-TBM, anti-tubular basement membrane; BSA, bovine serum albumin; CFA, complete Freund’s adjuvant; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; IF, immunofluorescence; LN, lymph node; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RTA, renal tubular antigen (basement membrane); SAS, saturated ammonium sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SRTA, collagenase-solubilized renal tubular antigen (basement membrane).
used to advantage in the process of identifying or selecting relevant α-TBM antibodies.

The RTA used to induce α-TBM disease consists of >15 proteins, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (unpublished observations). In our approach to the isolation of the relevant nephritogenic antigen we made the following assumptions: (a) a single protein in RTA could be responsible for causing disease, (b) the nephritogenic determinant would be TBM specific, (c) this moiety would exist in BN rat TBM but not in LEW rat TBM, and (d) an α-TBM monoclonal antibody (mAb) to such a determinant could reversibly bind it under appropriate experimental conditions. Consequently, we used immunoaffinity chromatography with a relevant α-TBM-mAb to isolate a single antigen that is capable of inducing α-TBM disease in susceptible hosts. Biochemical analyses reveal this antigen to be a noncollagenous glycoprotein. Because collagenase-solubilized RTA (SRTA), at an otherwise nephritogenic dose, is rendered innocuous with selective removal of this disease-causing moiety, we believe that the purified glycoprotein may represent the antigen of α-TBM disease. Immunoelectron microscopic studies have precisely localized this determinant along and adjacent to the tubular basement membrane. These data indicate that one glycoprotein is responsible for the induction of α-TBM disease in this model.

Materials and Methods

Animals. SJL and BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, ME. (SJL × BALB/c)F₁ mice were bred in our own facility. BN and LEW rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. Strain XIII guinea pigs were a gift from Ulrich Rudofsky, Kidney Disease Institute, New York State Department of Health, Albany, NY. Additional strain XIII guinea pigs were obtained from the University of Texas Veterinary Resource Division, Bastrop, TX. All animals were maintained by the Department of Laboratory and Animal Medicine, University of Pennsylvania School of Medicine.

Preparation of Renal Tubular Antigen. Rabbit RTA was isolated by a differential sieving technique (17). Highly enriched basement membrane fragments were sonicated, lyophilized, and stored at -70°C. SRTA was made from these lyophilized membranes using bacterial collagenase (CLS IV; Worthington, Division of Cooper Biomedical, Malvern, PA) digestion in the presence of protease inhibitors (18).

Immunofluorescence Analysis. Hybridoma culture supernatants were overlayed on cryostat sections of snap-frozen mouse or rat kidneys. After a 30 min incubation, sections were washed in phosphate-buffered saline (PBS), incubated for 30 min with fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG, washed again, and covered with glycerol containing phenyldiamine (19). Direct immunofluorescence (IF) of nephritic guinea pig kidneys was performed with FITC-conjugated anti-guinea pig IgG.

Monoclonal Antibody Production. Fusion techniques were adapted from those of McKearn (20) and Kennett (21). Fusion supernatants were screened using indirect IF, and subcloning was done in soft agar (22) or by limiting dilution (23). Ascites was produced in (SJL × BALB/c)F₁ mice after pristane priming. α-TBM mAb (anti-3M-1) was obtained using lymph node (LN) cells from an SJL mouse subcutaneously immunized with RTA in CFA. Anti-29M-1 mAb (TBM and glomerular basement membrane [GBM]–binding) was obtained in a separate fusion using LN cells from an SJL mouse also subcutaneously immunized with RTA in CFA.

Affinity Chromatography. 50% saturated ammonium sulfate (SAS) precipitates of mAb ascites were purified on a protein A–Sepharose CL-4B affinity column (Pharmacia Fine Chemicals, Piscataway, NJ). Purified anti-3M-1 and anti-29M-1 antibodies were then
coupled to Sepharose 4B (2 mg antibody/ml Sepharose). SRTA was passed over individual mAb affinity columns, and the bound protein was reverse eluted with 0.1 M citrate buffer in 3 M KCl, pH 2.5 into neutralizing buffer. Eluted aliquots of 100–200 μg of protein were dialyzed against 0.1 M phosphate buffer, pH 8.0 and concentrated under vacuum dialysis. Eluted protein from the anti-3M-1 and anti-29M-1 affinity columns are designated 3M-1 and 29M-1, respectively.

**Gel Electrophoresis.** 9% SDS-PAGE gels of pre-column SRTA and column eluates were performed by the slab technique of Laemmli (24). The gel bands were developed with silver stain (25).

**Biochemical Analyses.** Amino acid analysis was performed on protein samples hydrolyzed in 6 N HCl at 110°C for 24 h using a Beckman 121MB amino acid analyzer (Beckman Instrument, Inc., Fullerton, CA). Neutral sugars were determined by high pressure liquid chromatography on protein samples hydrolyzed with trifluoroacetic acid at 100°C for 5 h.

**Solid Phase Radioimmunoassay (RIA).** 25 μl of antigen (10 μg/ml) in 0.1 M phosphate buffer, pH 8, were added to individual wells of a 96-well, V-bottomed polyvinyl chloride microtiter plate (Cook Products/Dynatech Laboratories, Alexandria, VA) and were incubated overnight at 4°C. The wells were then blocked with 4% bovine serum albumin (BSA) for 1 h and washed; 25 μl of serially diluted anti-3M-1 ascites, anti-29M-1 ascites, or affinity-purified mouse IgG (as a specificity control) were added. After incubation at 22°C for 2 h, plates were washed and 125I-anti-mouse IgG was added for 2 h at 22°C. After thorough washing, the wells were cut and counted. Activity of wells lined with eluate was compared with that of wells lined with equivalent amounts of SRTA, column filtrate, and BSA.

**Induction of Disease.** Strain XIII guinea pigs receiving a single subcutaneous injection of 50 μg of antigen in CFA were sacrificed 18–22 d later. Kidneys (hematoxylin and eosin–stained sections) from these animals were examined for pathology, and the degree of tubulointerstitial disease assessed on a previously published (26), semiquantitative scale (0–4+). Direct IF (see above) was also performed.

**Immunoelectron Microscopy.** This methodology has been previously described in detail (27). Briefly, BN rat kidneys were fixed by in vivo formaldehyde perfusion. 8-μm frozen sections were mounted on albumin-coated slides and reacted with borohydride, followed by normal goat serum, primary mAb (or normal mouse serum), goat anti–mouse IgG, and mouse peroxidase-antiperoxidase complex, with appropriate PBS washes. Tissue was treated with OsO4, embedded in plastic, cut into ultrathin sections, mounted on uncoated grids, and photographed without any further staining.

**Results**

**Assessment of Anti-3M-1 Antibody Specificity by IF.** Indirect IF of anti-3M-1 mAb on normal SJL kidney sections revealed linear staining of the tubular perimeter corresponding to the region of the tubular basement membrane (Fig. 1A). While there was also staining of Bowman's capsule, intraglomerular structures themselves were devoid of IF, except for minor granular mesangial staining (Fig. 1A). This slight mesangial staining is identical to that seen by direct IF of normal SJL kidney sections (28). A similar pattern of IF with anti-3M-1 antibody was seen by indirect IF on normal BN rat kidney sections (Fig. 1B), whereas normal LEW rat kidney sections were negative (Fig. 1C). When anti-29M-1 mAb was used in indirect IF, in contrast to anti-3M-1, there was equivalent staining of both the TBM and the GBM. Further distinguishing anti-29M-1 from anti-3M-1 antibody was the ability of the former to stain both normal BN and LEW rat tubules to the same extent (data not shown). Thus, anti-3M-1 antibody
FIGURE 1. Indirect immunofluorescence of anti-3M-1 antibody on kidneys from (A) normal SJL mouse, (B) normal BN rat, and (C) normal LEW rat. Note intense staining of SJL and BN TBM zone and Bowman’s capsule, and absent GBM staining. LEW kidney was entirely negative.
appears to recognize both TBM and Bowman's capsule. Anti-3M-1 binds normal BN rat kidney, but not LEW rat kidney, an important distinguishing characteristic (5, 12, 14).

**Evaluation of Affinity Chromatography Eluates.** The material eluted from anti-3M-1-Sepharose 4B affinity columns typically represented ~0.05% of the starting renal TBM preparation. Pre-column SRTA, column filtrate, and eluate were analyzed in an indirect, solid phase RIA with anti-3M-1 (Fig. 2A). The column eluate was markedly enriched (four orders of magnitude) for the 3M-1 epitope, compared with pre-column SRTA. Conversely, column filtrate appeared to be relatively depleted of this determinant. To exclude the possibility that column eluate nonspecifically bound 3M-1 antibody, eluate-lined wells were reacted with anti-3M-1, anti-29M-1, or mouse IgG in the indirect RIA. Fig. 2B demonstrates
that column eluate (3M-1) was bound by anti-3M-1 antibody, but not by either anti-29M-1 or mouse IgG. Thus, the material eluted from the anti-3M-1 affinity column was specifically enriched for the 3M-1 epitope.

3M-1 was then further evaluated by SDS-PAGE. Fig. 3 demonstrates that silver-stained 3M-1 is predominantly composed of a 48,000 mol wt band, which was identical under both reduced (Fig. 3C) and nonreduced (D) conditions. For comparison, SDS-PAGE of collagenase (A) and pre-column SRTA (B) is shown. Both the absence of collagenase bands from 3M-1 and the heterogenous nature of pre-column SRTA are evident.

Amino acid analysis of 3M-1 (Table I) indicates that this protein is devoid of 3-hydroxyproline and hydroxylysine residues, and contains <6 residues per 1,000 of 4-hydroxyproline, suggesting that 3M-1 is noncollagenous in nature. Neutral sugar analysis (Table I) demonstrates significant amounts of glucose, and only traces of galactose, mannose, and fucose.

29M-1 antigen was purified as a control protein from an anti-29M-1 affinity column using pre-column SRTA as a starting material. Solid phase RIA analysis of the column eluate revealed strong reactivity with anti-29M-1 and SDS-PAGE showed two major bands of 29,000 and 45,000 mol wt (data not shown).

Evaluation of the Ability of the 3M-1 Glycoprotein to Induce α-TBM Disease. Strain XIII guinea pigs, a highly susceptible strain, were injected with 50 μg of various antigen preparations in CFA and were sacrificed 18–22 d later. Table II documents the nephritogenic nature of crude SRTA and the purified 3M-1 moiety. Fig. 4A demonstrates the extent of tubulointerstitial destruction after

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**Figure 3.** SDS-PAGE of the 3M-1 protein. (A and B) Bacterial collagenase and SRTA, respectively (both reduced); (C) reduced 3M-1; (D) unreduced 3M-1. The 3M-1 lanes reveal a predominantly 48,000 mol wt protein, with a few much fainter, higher molecular weight bands. 3M-1 is devoid of both bacterial collagenase contamination and most SRTA proteins.
**Table I**

*Biochemical Analysis of 3M-1*

| Amino acids       | Residues per 1,000 | Neutral sugars | Percent of protein |
|-------------------|--------------------|----------------|-------------------|
| Cysteic acid      | 11.32              | Glucose        | 6-8               |
| 3-Hydroxyproline  | 0.00               | Galactose      | Trace             |
| Methionine sulfoxide | 2.64            | Fucose         | Trace             |
| 4-Hydroxyproline  | 5.88               | Mannose        | Trace             |
| Aspartic acid     | 49.58              |                |                   |
| Threonine         | 15.27              |                |                   |
| Serine            | 39.41              |                |                   |
| Glutamic acid     | 139.38             |                |                   |
| Proline           | 81.20              |                |                   |
| Glycine           | 161.80             |                |                   |
| Alanine           | 154.09             |                |                   |
| Cystine           | 0.00               |                |                   |
| Valine            | 49.22              |                |                   |
| Methionine        | 0.00               |                |                   |
| Isoleucine        | 45.30              |                |                   |
| Leucine           | 93.44              |                |                   |
| Tyrosine          | 0.51               |                |                   |
| Phenylalanine     | 28.94              |                |                   |
| Hydroxylysine     | 0.00               |                |                   |
| Lysine            | 52.44              |                |                   |
| Histidine         | 20.41              |                |                   |
| Arginine          | 49.16              |                |                   |

**Table II**

*Induction of Disease in Strain XIII Guinea Pigs*

| Antigenic preparation | n | Pathologic score* |
|-----------------------|---|-------------------|
| 3M-1                  | 4 | 2.9 ± 0.6         |
| SRTA                  | 3 | 2.2 ± 0.4         |
| Column filtrate        | 3 | 0.0 ± 0.0         |
| 29M-1                 | 3 | 0.0 ± 0.0         |

All guinea pigs received 50 µg of the designated preparation in CFA, subcutaneously. Animals were sacrificed 18-22 d later and their kidneys processed as described in Materials and Methods.

* Hematoxylin and eosin-stained sections were evaluated on a scale of 0-4 describing the extent of cortical tubulointerstitial involvement with mononuclear cell infiltrates.

**Electron Immunohistochemical Localization of the 3M-1 Determinant.** These data
FIGURE 4. Histologic and IF evaluation of strain XIII guinea pig kidney 18 days after injection of 5 M-1 (50 μg) in CFA. (A) Light microscopy of hematoxylin and eosin-stained section. There is marked tubular cell destruction and atrophy with accompanying cystic dilatation of tubular lumens, and surrounding interstitial infiltrates of mononuclear cells. The glomerulus is intact. (B) Direct IF demonstrates TBM zone staining amidst a distorted tubulointerstitial architecture.
confirm and extend observations by indirect IF on the distribution of 3M-1. The antigen was demonstrated on TBM (Fig. 5A), predominantly in the interstitial aspect of these structures (Fig. 5, C and D). The inner aspects of the TBM (closer to the tubular cell) were consistently negative (Figs. 5, C and D). Large, interstitial collagen fibers were often, but not always, coated with the antigen (Fig. 5, A, C, and D). The interstitial aspect of the arteriolar smooth muscle basement membrane was occasionally positive (data not shown). Bowman’s capsule was consistently positive with the antibody (Fig. 5A), reacting in a similar pattern to that of TBM; i.e., the interstitial aspects were positive, whereas the glomerular aspects were negative. The GBM and the mesangial matrix were consistently negative (Fig. 5, A and B), confirming one of the original criteria for relevance of anti-3M-1 antibody—that it is TBM specific and unreactive with GBM.

Altogether, these data document that the 3M-1 antigen is primarily located in the interstitium and on the interstitial aspect of the TBM. Furthermore, 3M-1 is not a demonstrable glomerular antigen.

Discussion

Observations from both clinical and experimental studies document the involvement of destructive immunopathogenic mechanisms in the tubulointerstitial nephropathies (reviewed in 29 and 30). Attempts to elucidate relevant immunologic processes leading to interstitial damage have relied heavily on animal models that use complex antigen preparations to induce pathologic renal lesions. The availability of a single nephritogenic antigen would obviously permit a clearer understanding of afferent, regulatory, and efferent factors in the immune response. The present investigation describes the isolation of such a molecule.

3M-1 is a 48,000 mol wt glycoprotein derived from bacterial collagenase-digested TBM. This antigen is capable of inducing α-TBM antibodies and severe interstitial nephritis in susceptible hosts. Because the 3M-1 antigen lacks hydroxylysine and 3-hydroxyproline residues, and contains a very low amount of 4-hydroxyproline, it probably derives from noncollagenous glycoproteins that have been previously described (31) as constituents of basement membranes of various origins. Interestingly, the carbohydrate portion of 3M-1 is comprised of glucose, largely to the exclusion of other frequently encountered sugar moieties, particularly mannose and galactose. Although uncommon, similar glycoproteins have been isolated from renal tissue by other investigators. Shibata and Nagasawa (32), for example, have described a nephritogenic glycoprotein capable of causing glomerular disease that, similarly, possessed glucose as its predominant neutral
sugar. Their method of antigen preparation, IF localization, and induction of a proliferative glomerulonephritis all distinguish it from 3M-1. Finally, the possibility that 3M-1 is the nephritogenic moiety of rabbit RTA which causes α-TBM disease is suggested by the demonstration that selective depletion of 3M-1 from a pre-column SRTA preparation results in the loss of its nephritogenic capabilities.

Several previous studies have explored the molecular nature of basement membranes in general, TBM in particular, and the relevant component of RTA that produces α-TBM disease. All basement membranes have a common structural backbone: type IV collagen. The amino- and carboxy-terminal procollagen peptides of this collagen consist of noncollagenous sequences with intrachain or interchain disulfide bonds. Basement membranes, including those found in the kidney, also have several noncollagenous components: entactin, heparan sulfate proteoglycan, and laminin, which are integrated into the basement membrane via covalent and noncovalent bonds (31). Thus noncollagenous antigens, including 3M-1, could derive either from the noncollagenous domains of procollagen, or from other noncollagenous components. However, the present study demonstrates that the ultrastructural localization of 3M-1 is distinctively different from that of type IV collagen (33, 34), entactin (27), heparan sulfate proteoglycan (34), and laminin (33, 35). In addition to the different localization within basement membranes, all these known components of basement membranes are present in the GBM. This is in contrast to 3M-1, which is not demonstrable in GBM. These considerations suggest that 3M-1 is either a previously unknown component of basement membranes, or an interstitial antigen that, in vivo, is in close apposition to some basement membranes. The ultrastructural localization favors the second possibility.

Several groups have investigated the glycoprotein nature of the TBM per se. Ferwerda et al. (36) isolated TBM glycoproteins after collagenase digestion of bovine RTA. Two distinct glycoproteins were identified, one of which was TBM specific, the other of which had epitopes shared by both TBM and GBM. This parallels our experience with 3M-1 and 29M-1, the former being associated with the TBM and not GBM, and the latter present in both TBM and GBM. Butkowski et al. (37) used SDS solubilization to study the glycoprotein-rich component of rabbit TBM. By SDS-PAGE analysis, they documented at least 18 distinct moieties in this fraction, ranging in molecular weight from 18,500 to 1,000,000. In these studies the potential pathogenicity of individual proteins was not assessed, nor was the location of these determinants evaluated by electron microscopy. Paul and Carpenter (38) analyzed the antigenic profile of the rat proximal tubular basement membrane using serologic patterns of reactivity. They also were able to discern at least two distinctive determinants, although these were not isolated nor further characterized. Interestingly, neither was present in LEW rat TBM, and both appeared to be encoded outside of the major histocompatibility complex (MHC). These observations are consistent with what is known of the target antigen of α-TBM disease. LEW rats lack this determinant (and are consequently protected from the induction of α-TBM disease), and genetic mapping suggests that the relevant locus exists on a non-MHC chromosome (12). Very recent data (13) indicate that the gene for the TBM nephrito-
few investigations have attempted to isolate purified antigens capable of inducing autoimmune renal disease. Wakashin et al. (39), however, have reported on a nephritogenic antigen in α-TBM disease. They digested human TBM with trypsin, and used a series of physicochemical methods to purify a 30,000 mol wt determinant that was capable of inducing α-TBM antibody-associated interstitial nephritis in outbred goats and BALB/c mice. Three considerations make these observations somewhat unusual. First, because trypsin digestion was used, one cannot exclude the possibility that this antigen was enriched for collagenous components, rather than glycoprotein; unfortunately, amino acid analysis and immunoelectron microscopy were not provided. Previous experience with renal basement membrane collagen suggests that this basement membrane constituent is generally not pathogenic (unpublished observations). Secondly, the TBM antigen isolated by Wakashin et al. (39) caused, in addition to interstitial nephritis, a proliferative glomerulonephritis. Such glomerular lesions have not been seen as part of the spectrum of classical α-TBM disease, and raise the issue of exactly how their moiety relates to the traditional experimental model of tubulointerstitial disease. Lastly, it is surprising that C57BL/6 mice immunized with their TBM antigen did not mount an α-TBM antibody response. Previous investigations (4, 28) using tubular antigen have documented α-TBM antibody production among all mouse strains thus far tested (including C57BL/6), regardless of susceptibility to nephritic disease. Using a different approach, Zanetti and Wilson (40) have suggested that, in the rat model of α-TBM disease, a 42,000 mol wt, collagenase-solubilized component of bovine and BN rat TBM may be a relevant moiety for disease induction. The nephritogenic capabilities of this determinant, however, were not directly analyzed.

Finally, immunoelectron microscopy has revealed the fine detail of 3M-1 distribution. 3M-1 is predominantly localized to the most lateral aspects of the TBM, bordering, and adjacent to the interstitial region; the glomerulus is devoid of such determinants. As discussed above, this unique distribution distinguishes 3M-1 from other known protein constituents of renal basement membranes, including type IV collagen (33, 34), entactin (27), heparan sulfate proteoglycan (34), and laminin (33, 35). Consequently, it would appear that the 3M-1 epitope identifies a unique renal interstitial and/or basement membrane–associated glycoprotein.

**Summary**

Using monoclonal antibody affinity chromatography, we isolated a 48,000 mol wt, glucose-rich glycoprotein (3M-1) from collagenase-solubilized rabbit renal tubular basement membrane (SRTA). The purified 3M-1 protein is noncollagenous, and is capable of inducing anti-TBM (tubular basement membrane) antibodies and interstitial nephritis in susceptible hosts. Further, when SRTA, at a normally nephritogenic dose, was selectively depleted of 3M-1, it lost its ability to induce disease. As shown by immunofluorescent techniques, 3M-1 appears to be localized on rodent TBM to the exclusion of the glomerular basement membrane, but was lacking in the TBM of the LEW rat, a strain devoid of the...
relevant antigen of anti-TBM disease. Immunoelectron microscopy revealed that 3M-1 was associated with the most lateral aspect of the TBM, which borders, and lies in the interstitium. These results indicate that 3M-1 is the nephritogenic antigen producing experimental anti-TBM disease.

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