CLONOTYPIC HETEROGENEITY IN EXPERIMENTAL INTERSTITIAL NEPHRITIS

Restricted Specificity of the Anti–Tubular Basement Membrane B Cell Repertoire Is Associated with a Disease-modifying Crossreactive Idiotype

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Recent efforts in our laboratory have concentrated on autoimmune mechanisms producing an experimental interstitial nephritis called anti–tubular basement membrane (anti-TBM)[1] disease (1). Anti-TBM disease is induced in rodents after an immunization with renal tubular antigen (RTA) in adjuvant. Susceptible animals expressing the endogenous antigen (2) develop antibodies to the tubular basement membrane (anti-TBM-Ab) (3), a complex nephritogenic T cell repertoire (4), active fibrogenesis (5), interstitial cell infiltrates, and progressive renal failure. Such renal lesions can also spontaneously develop in patients, resulting in progressive renal insufficiency (6–8). Both the nephritogenic antigen (3M-1) in the experimental model and the target antigen of human disease are crossreactive moieties of similar size (48,000 M_r) (9). This association is seemingly unique among immune-mediated renal lesions, and supports the notion that experimental anti-TBM disease may be analogous to the human condition.

With the ability to now isolate the active nephritogenic moiety of anti-TBM disease, we have extended our previous observations to provide a detailed analysis of its paratypic recognition by the autoimmune B cell repertoire. A library of 22 rat anti-TBM mAbs, each specific for the nephritogenic antigen (3M-1), was constructed for this analysis from five separate fusions. Their characterization revealed an anti-TBM (anti-3M-1) B cell population that consists of ~58 distinct clones that express at least three V_H gene families, widely variable affinities, a broadly crossreactive idiotype (IdX), but single specificity for an immu-

[1] Abbreviations used in this paper: Ab, antibodies; anti-TBM, anti–tubular basement membrane; BN, Brown Norway; DTH, delayed-type hypersensitivity; FPLC, fast-performance liquid chromatography; IdX, crossreactive idiotype; RTA, rabbit renal tubular antigen; SRTA, collagenase-solubilized renal tubular antigen.

nodominant epitope. A representative rat anti-TBM mAb, furthermore, was capable of inhibiting >80% of the binding of polyclonal human anti-TBM antisera. Finally, we demonstrate that polyclonal anti-IdX-Ab can be used therapeutically, not only to prevent the induction of experimental disease, but also to arrest the progression of the renal lesion.

Materials and Methods

Animals

Brown Norway (BN) and Lewis rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA.

Antigens

Rabbit RTA was prepared by differential sieving (10). Collagenase digestion of RTA to produce collagenase-solubilized renal tubular antigen (SRTA) was accomplished with bacterial collagenase (CLS IV; CooperBiomedical, Inc., Malvern, PA) in the presence of protease inhibitors (11). Rabbit and human 3M-1 were isolated from SRTA using affinity chromotography (2, 9).

Monoclonal Antibodies

Draining lymph node cells were harvested on day 18 from Brown Norway rats immunized on days 0 and 14 with RTA in CFA, and were fused to Sp2 cells using standard hybridoma technology (12). Acidic supernatants of wells with growing hybridomas were screened by indirect immunofluorescence on Brown Norway rat kidney sections with fluorescein-conjugated anti–rat IgG, and those that displayed linear staining of the TBM without glomerular staining were considered preliminary positives. These presumptive positives were then examined on kidney sections from Lewis rats, a strain generally acknowledged to lack the target antigen of anti-TBM disease (3, 13, 14). If negative on these sections, they were doubly subcloned at 0.5 cells/well.

Characterization of mAbs

Subclass: Light Chain. This was determined by Ouchterlony immunodiffusion of serum-free (HB101; NEN Research Products, Boston, MA) hybridoma supernatants with rat IgG subclass or K light chain antisera (Gateway Immuno-Sera Company, St. Louis, MO).

Quantitation of Anti-TBM mAb. Because all anti-TBM mAb were of the IgG1 or IgG2a subclass, two reference hybridomas representing each subclass were used to make ascites in pristane-primed nude mice. The respective mAbs were then purified by fast-performance liquid chromatography (FPLC, Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and quantitated for use in constructing a standard curve. Solid-phase RIAs were performed by serially diluting the reference and test anti-TBM mAbs into the wells of polyvinyl chloride (PVC) microtiter plates (Dynatech Laboratories, Inc., Alexandra, VA) in HB101 medium. The wells were blocked with 4% BSA and developed with affinity-purified ^{125}I-goat anti–rat IgG (Zymed Laboratories, San Francisco, CA). Unknown anti-TBM mAb concentrations were determined by extrapolation to the appropriate (either IgG1 or IgG2a) standard curve.

Specificity and Relative Affinity. Recognition of 3M-1 was also evaluated by a solid-phase RIA in which serial dilutions of HB101 hybridoma supernatants were incubated in PVC wells lined with constant amounts (250 ng/well) of rabbit 3M-1. The reaction was developed with affinity-purified ^{125}I-goat anti–rat IgG. Relative affinity was determined by comparing quantities of mAb required to achieve 50% of maximum binding to 3M-1. Two monoclonals (56R-7 and 57R-2) had been previously assigned binding affinity constants (Shih, W. Y., M. D. Clayman, C. J. Kelly, W. Hines, and E. G. Neilson, submitted for publication) after formal Scatchard plot analysis using the method of Frankel and Gerhard (15).

Isoelectric Focusing Patterns. Anti-TBM mAbs of the same subclass and light chain,

which had relative affinities within 25% of each other, were assigned to one of five arbitrary groups for isoelectric focusing to determine possible identities. These candidate anti-TBM mAbs were FPLC purified and then run in a vertical 5.5% polyacrylamide gel using LKB ampholytes to achieve a pH range of 3.5–10 (16). Focused gels were then labeled with affinity-purified ^{125}I–mouse anti–rat IgG and developed by autoradiography (17).

Quantitative Estimation of the Anti-TBM B Cell Repertoire.   The identical pair formula, as adapted by Briles and Carroll (18), was used to provide a quantitative estimate of the anti-TBM B cell repertoire. This formula states: $R = [n(n - 1)/2]/ [\sum_{i=1}^{m} a_i(a_i - 1)/2]$,

where R is the number of distinct TBM-specific B cells; n is the total number of relevant monoclonal anti-TBM antibodies (anti-TBM mAb); a_i is the number of anti-TBM mAbs with identity i; i is a particular anti-TBM mAb that shares identity wtih one or more other anti-TBM mAbs; and m is the total number of such identical groups.

Paratypic Analysis.   The epitopic specificity of each anti-TBM mAb was assessed by competitive inhibition RIA. Increasing concentrations of different unlabeled anti-TBM mAbs were admixed with constant amounts of a labeled anti-TBM mAbs (^{125}I–57R-2; radiolabeled by the chloramine T method [19]) and the mixture was added to PVC microtiter plates that had been previously lined with 3M-1 (250 ng/well). After 2 h of incubation, plates were washed and wells were counted in a gamma counter.

V_H Analysis.   Hybridomas were grown to 10^8 cells, washed twice with sterile PBS, and their RNA was extracted by lithium chloride precipitation (20) or homogenization in guanidinium thiocyanate (21). RNA was then separated on 1% agarose in 2.2-M formaldehyde gels, transferred to nylon filters, and analyzed with three different murine V_H probes. These were pVHS107 from the S107 family (22), pVHQ52N from the Q52 family (22), and pVhSAPC-15 from the 7183 family (22). Probes were nick translated with [^{32}P]dCTP and were added to hybridization buffer at a concentration of 10^6 cpm/ml. After low- and high-stringency washes, filters were exposed to XAR-5 film (Eastman Kodak Co.) in cassettes containing Cronex Lightning-plus intensifying screens (DuPont Co., Wilmington, DE) at −70°C for 3–5 d and were then developed.

Preparation of Anti-IdX-Ab

10 anti-TBM mAbs were initially used as immunogens to potentially induce an anti-IdX antisera. Only one (53R-4) was found to be inductive (see Results). This antisera was prepared in the following way: anti-TBM mAb 53R-4 was FPLC purified from ascites, mixed with CFA, and used to immunize an outbred rabbit subcutaneously at 0 (200 μg 53R-4), 2 (100 μg), and 4 wk (100 μg). At 5 wk, harvested serum was exhaustively adsorbed over a normal BN serum (50%-saturated ammonium sulfate precipitate) Sepharose 4B (Pharmacia Fine Chemicals) affinity column to remove reactivity with Ig constant region or framework determinants. Adsorbed anti-53R-4 antisera was titered against 53R-4 in a solid-phase RIA. Reactivity with BN IgG and normal rabbit serum reactivity with 53R-4 were also determined as specificity controls. A competitive inhibition RIA was then performed in PVC microtiter plates lined with 250 ng/well of 53R-4. 25-μl aliquots consisting of equal volumes of increasing concentrations of various FPLC-purified anti-TBM mAbs and a 1:1,500 dilution of rabbit anti-53R-4 antisera (which represented twice the 50% maximum binding value) were then applied to the PVC plate and developed with ^{125}I–anti–rabbit IgG.

Treatment of Anti-TBM Disease with Anti-IdX Antisera

0.5 ml of anti-IdX antisera (anti-53R-4) was administered intravenously to four BN rats 2 d before and on the day of immunization with 2 mg of RTA/CFA. Another group of four BN rats also received 0.5 ml of IdX antisera on days 14 and 16 after immunization. Control groups consisted of animals immunized with RTA/CFA or CFA alone without any antisera administration, and RTA/CFA-immunized animals that received normal rabbit serum instead of anti-IdX antisera. Two animals from the RTA/CFA control group were also killed at 14 d, and their kidneys were examined to establish baseline

histology. All of the other animals in all groups were killed on day 27. Kidneys were preserved in 10% buffered formalin, set in paraffin blocks, sectioned, and stained with hematoxylin and eosin. Sections were read without knowledge of experimental groups. The intensity of the cortical interstitial mononuclear cell infiltrate was graded on a previously published (23) scale of 0 to 4+: 0, no infiltrate; 1+, <25% of cortex involved; 2+, 25–50% of cortex involved; 3+, 50–75% of cortex involved; 4+, >75% cortex involved. Delayed-type hypersensitivity (DTH) reactions to SRTA and purified protein derivative were measured on the day of sacrifice, 24 h after footpad challenge with 25 μ g of SRTA or PPD in 25 μl of PBS. Swelling was determined by a spring-loaded engineer's micrometer (Schlesingers for Tools Ltd., Brooklyn, NY) and was read as incremental swelling (in 10^{-3} in) compared with PBS-injected controls (4). Serologic anti-TBM-Ab (anti-3M-1) responses were quantitated by solid-phase RIA (20).

Specificity of anti-TBM-Ab from Human Antisera

PVC plates were lined with human 3M-1 (250 ng/well) and reacted with serial dilutions of human anti-TBM-Ab (9). Reactions were developed with affinity-purified ^{125}I–anti–human IgG. The serial dilution representing 50% of maximum binding was then used to examine the ability of increasing concentrations of a rat anti-TBM mAb (56R-9) to competitively inhibit the binding of human anti-TBM-Ab to 3M-1. In this assay, aliquots of the human antiserum dilution representing twice the 50% maximum binding value were admixed with equal volumes of increasing concentrations of the rat anti-TBM mAb for 30 min and then were applied to PVC plates previously lined with human 3M-1. Reactions were developed with ^{125}I–anti–human IgG and counted in a gamma counter.

Results

Library of Rat Anti-TBM mAb.   22 IgG anti-TBM mAb were isolated from five different fusions. As shown in Table I, 20 of the 22 IgG possessed κ light chains and all were either of the IgG1 or IgG2a subclass. By indirect immunofluorescence, these all demonstrated linear TBM staining on BN rat kidney sections, but were negative on Lewis rat kidney (data not shown). Each was reactive with 3M-1 in a solid-phase RIA. There was an exact correlation between immunofluorescent positivity and 3M-1 reactivity. The relative binding affinities, expressed as the quantity of mAb required to achieve 50% of maximum binding to 3M-1 in a solid-phase RIA, spanned a broad range (Table I). To place these data in perspective, formally determined binding affinity constants for 56R-7 and 57R-2 are 6 × 10^6 liters/mol and 2 × 10^8 liters/mol, respectively (Shih, W. Y., M. D. Clayman, C. J. Kelly, W. Hines, and E. G. Neilson, submitted for publication). Fig. 1 shows the binding curves of several representative anti-TBM mAbs to 3M-1.

Epitopic Specificity.   The ability of unlabeled anti-TBM mAb to competitively inhibit binding of ^{125}I–57R-2 to 3M-1 was assessed by solid-phase RIA (Fig. 2). In this representative figure, all anti-TBM mAb, over the dosage range examined, inhibited >50% of ^{125}I–57R-2 binding, whereas BN rat IgG did not substantially inhibit at all. In fact, all 22 anti-TBM mAbs were tested and all but one of these displayed comparable competitive inhibition curves. Anti-TBM mAbs 56R-1 inhibited somewhat less, to a maximum of 40% (data not shown). These data collectively suggest that nearly all of the anti-TBM mAbs from five separate fusions may be directed to an immunodominant epitope on 3M-1.

Quantitation of the anti-TBM B Cell Repertoire.   The identical pair formula (17) predicts that the number of distinct members of a specific clonotype is inversely

TABLE I

Library of Anti-TBM mAbs

Anti-TBM mAb	Phenotypes*		Supernatant[‡] concentration	Relative 3M-1[§] binding affinity
	Light chain	Isotype		
			µg/ml	
53R-1	κ	IgG2a	28.2	2.75
53R-2	κ	IgG1	2.7	>68.00
53R-3	—[∥]	IgG1	6.0	>150.00
53R-4	κ	IgG2a	15.6	2.75
53R-5	κ	IgG2a	18.6	2.50
55R-1	κ	IgG2a	20.0	0.74
56R-1	κ	IgG1	31.0	19.00
56R-2	κ	IgG2a	30.4	2.85
56R-3	κ	IgG2a	25.2	4.00
56R-6	κ	IgG2a	78.0	1.70
56R-7	κ	IgG1	17.2	400.00
56R-9	κ	IgG1	4.8	0.22
56R-11	κ	IgG2a	304.0	>400.00
56R-12	κ	IgG2a	22.8	30.00
57R-1	κ	IgG2a	33.0	0.73
57R-2	κ	IgG1	8.0	0.33
57R-3	κ	IgG1	25.8	0.50
57R-4	—[∥]	IgG1	11.2	<0.07
57R-6	κ	IgG2a	33.6	0.95
57R-7	κ	IgG2a	75.6	3.40
57R-8	κ	IgG1	34.8	<0.21
Cl-12	κ	IgG1	7.2	0.73

* Light chain and isotype were determined by Ouchterlony immunodiffusion.
‡ Supernatant concentration was determined in a solid-phase RIA.
§ Quantity of mAb (in ng) necessary to achieve 50% of maximum binding to 3M-1 in a solid-phase RIA.
∥ Lack of recognition by anti-K antisera.

proportional to the likelihood of finding identical anti-TBM mAbs from different hybridomas. This was used to estimate the size of the anti-TBM B cell repertoire. For anti-TBM mAb that expressed the same light chain and subclass and had similar binding curves to 3M-1, isoelectric focusing was performed as a means of determining identity. 13 anti-TBM mAb were placed in five arbitrary groups of apparent similarity and their isoelectric points were determined by electrophoresis. Three of four anti-TBM mAb in group 1 and 2 of three antibodies in group 2 appeared to have identity. Fig. 3 illustrates the focusing patterns for groups 2 and 5. Thus, the identical pair formula estimates the size of the anti-TBM B cell repertoire at ~58 distinct clones, of which 19 were represented in this report.

Analysis of Hybridoma V_H Genes. Evaluation by Northern hybridization of six hybridomas with probes to three murine V_H gene families known to crosshy bridize strongly with different rat V_H genes indicated (Table II) that RNA from two hybridomas hybridized with the Q52 family (an IgM-sized band also hybrid-
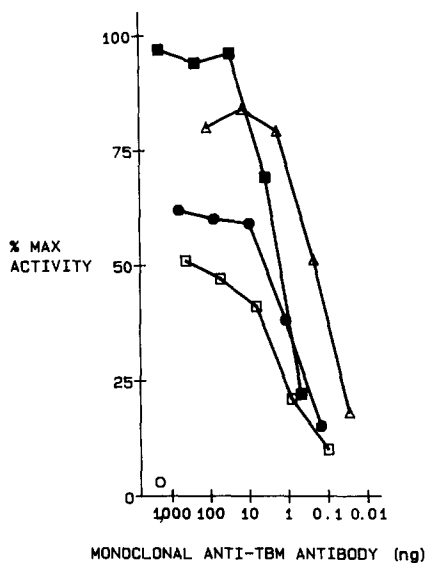
FIGURE 1. Solid-phase RIA of representative anti-TBM mAbs binding to 3M-1. HB101 supernatants, containing known amounts of anti-TBM mAb, were serially diluted and added to PVC microtiter plates lined with 250 ng/well of 3M-1. The reaction was developed with ^{125}I–anti-rat IgG. (—■—) anti-TBM mAb 56R-6; (—△—) anti-TBM mAb 56R-9; (—●—) anti-TBM mAb 56R-3; (—□—) anti-TBM mAb 56R-7; (O) media control. Maximum cpm in this assay was 4,666.

ized in hybridoma 56R-3); RNA from two hybridomas hybridized with the 7183 family (with borderline recognition of a third); and RNA from two other hybridomas did not hybridize with any of these V_H probes. A representative Northern blot is shown in Fig. 4. The IgM-sized RNA band from 56R-3 is presumed to be from a nonproductive Igh rearrangement because all anti-TBM mAb in this study have been shown to be of the IgG class (Table I). FPLC analysis of supernatants from 56R-3 did not reveal any IgM-sized protein (data not shown), indicating that the protein product of this larger mRNA is not cosecreted with the
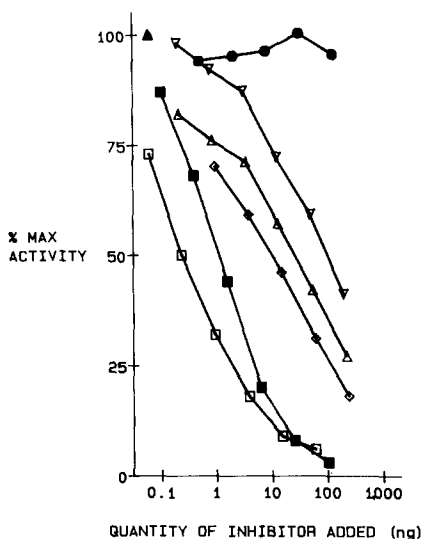

FIGURE 2. Competitive inhibition of ^{125}I–57R-2 binding to 3M-1 by representative "cold" anti-TBM mAb. Serial dilutions of inhibitors (anti-TBM mAb or BN rat IgG) were admixed with equal volumes of ^{125}I–57R-2, and aliquots were added to 3M-1-lined PVC microtiter wells. ^{125}I–57R-2 reactivity with an irrelevant antigen (BSA) was <1% of maximum. (▲) Uninhibited ^{125}I-57R-2. Inhibitors: (—●—) normal BN IgG; (—■—) 57R-2; (—◇—) 56R-6; (—▽—) 53R-4; (—△—) 56R-7; (—□—) 56R-9. Maximum cpm in this assay was 25,599.
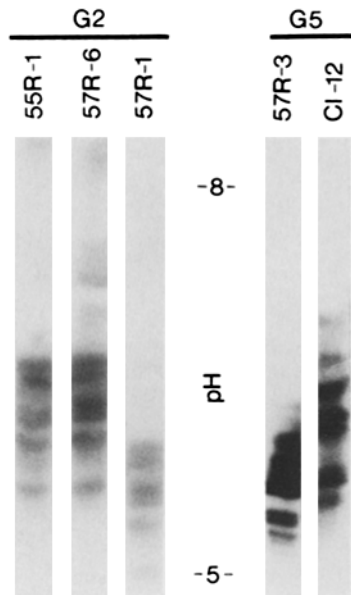
FIGURE 3. Isoelectric focusing patterns from two groups (G2 and G5) of anti-TBM mAb considered to have potential identity. G2: 55R-1 and 57R-6 appear to be identical, but are different from 57R-1. G5: 57R-3 and Cl-12 do not appear to be similar.

IgG. These data collectively suggest, therefore, the existence of at least three different V_H region gene families for the hybridomas examined. While two hybridomas of widely differing affinities (56R-7 and 57R-2) used V_H gene segments from the same family (Q52), three hybridomas (56R-3, 56R-7, and 57R-2) that shared a crossreactive idiotype (see below) were derived from two different V_H families (Q52 and 7183).

Shared Paratypic Specificity. Previous work (9) had demonstrated that the nephritogenic antigen of experimental anti-TBM disease was crossreactive with the target antigen of human anti-TBM disease. Given the finding of a presumed immunodominant epitope on rabbit 3M-1, we tested the possibility that a similar crossreactive epitope might exist on human 3M-1, and also that the serological anti–human 3M-1 response might also focus to this determinant. Consequently,

TABLE II

Analysis of IgG Hybridoma V_H Genes

Hybridoma*	IdX	V_H gene family		
		Q52	7183	S107
57R-1	−	−	−	−
57R-2	+	+	−	−
53R-3	ND	−	+	−
56R-7	+	+	±	−
56R-3	+	+(IgM)	+	−
57R-6	ND	−	−	−

* Hybridoma RNA was blotted and analyzed with labeled DNA probes specific for the indicated gene families.
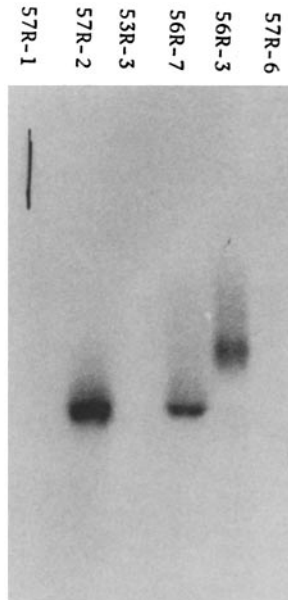
FIGURE 4. Representative Northern blot of hybridoma RNA. DNA probe pVhQ52N (from the Q52 family) was nick translated and hybridized to Northern blotted hybridoma RNA as shown. There is recognition of IgG-sized RNA from both 56R-7 and 57R-2. Higher molecular weight RNA (presumed IgM) from 56R-3 is also recognized.

we assessed the ability of 56R-9 to competitively inhibit the binding of human anti-TBM antisera (from a patient with spontaneous anti-TBM disease and renal failure) (9) to human 3M-1. As shown in Fig. 5, 56R-9 is capable of inhibiting >80% of human anti-TBM antisera reactivity with human 3M-1. Purified BN IgG does not inhibit at all.

Idiotypic Analysis. 10 anti-TBM mAbs were FPLC purified from ascites and were used to riase rabbit antiidiotypic antisera. Nine of these antisera only recognized private idiotypes on their respective immunogen. One of them, however, rabbit anti-53R-4 idiotype, could be competitively inhibited by five of
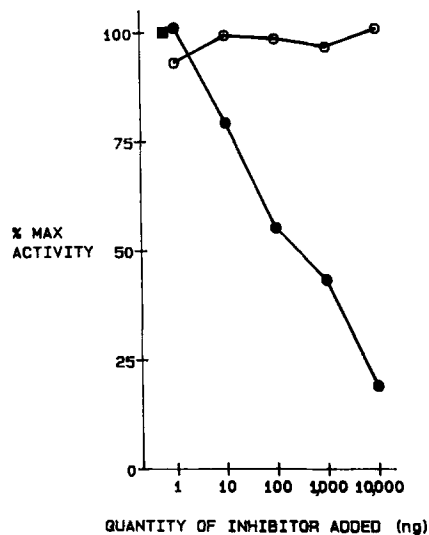

FIGURE 5. Epitopic focusing of polyclonal human anti-TBM antisera. Serial dilution of inhibitors (56R-9 [—●—] or BN rat IgG [—O—]) were admixed with human anti-TBM antisera (1:1,000 final dilution) and added to human 3M-1-lined PVC microtiter wells in a solid-phase RIA. The reaction was developed with ^{125}I–anti–human IgG. (—■—) Uninhibited human anti-TBM antisera. The specificity of this antisera for the target antigen of human anti-TBM disease (human 3M-1) has been previously established (9). Maximum cpm in this assay was 7,588.
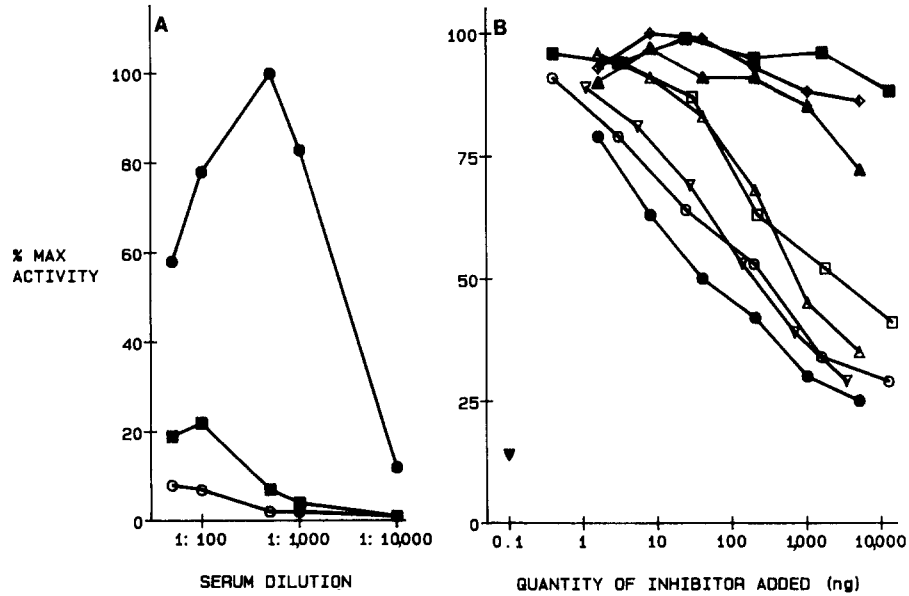
FIGURE 6. (A) Specificity of anti-(53R-4 idiotype) antiserum. Appropriately adsorbed rabbit anti-(53R-4 idiotype) antiserum or normal rabbit serum was serially diluted and reacted with 53R-4 or BN rat IgG-lined wells (250 ng/well) in a solid-phase RIA. The reaction was developed with ^{125}I–anti–rabbit IgG. (——●——) Rabbit anti-(53R-4 idiotype) antiserum vs. 53R-4; (——■——) rabbit anti-(53R-4 idiotype) antiserum vs. BN rat IgG; (——○——) normal rabbit serum vs. 53R-4. Maximum cpm in this assay was 35,833. (B) Rabbit anti-(53R-4 idiotype) antiserum recognition of a cross-reactive idiotype. In a competitive inhibition RIA, wells were lined with 53R-4 (250 ng/well) and were reacted with an admixture of serially diluted inhibitor and rabbit anti-(53R-4 idiotype) antiserum (1:3,000 final dilution). The reaction was developed with ^{125}I–anti–rabbit IgG. Inhibitors: (——■——) BN IgG: (——●——) 53R-4; (——○——) 56R-3; (——◇——) 57R-1; (——□——) 56R-7; (——▽——) 57R-2; (——△——) 56R-9; (——▲——) 53R-1; (——▼——) uninhibited rabbit anti-(53R-4 idiotype) antiserum recognition of BN IgG. Maximum cpm in this assay was 13,811.

seven randomly selected anti-TBM mAb (Fig. 6), whereas BN IgG did not inhibit at all. These data suggest that 53R-4 expresses an IdX shared by other anti-TBM-Ab in the B cell repertoire.

Treatment of anti-TBM Disease with anti-IdX Antisera. The ability of an anti-IdX-Ab (rabbit anti-53R-4) to inhibit the pathogenesis of experimental anti-TBM disease was examined at two time points. As shown in Table III, BN rats (group 3) that received anti-IdX-Ab in the periimmunization period (2 d before and on the day of immunization) had essentially no interstitial infiltrates when killed 4 wk later (group 3 average pathology score was 0.3). In contrast, animals that received either no treatment or normal rabbit serum developed severe interstitial nephritis with average pathologic scores of 3.9 (group 1) and 3.5 (group 2), respectively. The average pathology scores of untreated animals (group 1b) was 1.8 at 2 wk compared with 3.9 in group 1 rats at 4 wk. Animals that received 0.5 ml of anti-IdX-Ab on days 14 and 17 (group 5) had average pathologic scores of 1.6 at 4 wk, whereas animals treated with normal rabbit serum (group 4) progressed to an average pathologic score of 3.8. Thus, anti-IdX-Ab was both

TABLE III
Treatment of Anti-TBM Disease with Anti-IdX Antisera

Group	n	Immunization (Day 0)	Treatment*		Pathology scores‡	DTH$_{SRTA}$§	DTH$_{PPD}$
			Day -2/0	Day 14/17			
1	4	RTA/CFA	—	—	3.9 ± 0.1	23.0 ± 4.1	31.8 ± 0.8
1b	2	RTA/CFA[I]	—	—	1.8 ± 0.3	—	—
2	4	RTA/CFA	NRS	—	3.5 ± 0.3	26.8 ± 4.8	29.8 ± 1.0
3	4	RTA/CFA	Anti-IdX	—	0.3 ± 0.3	5.5 ± 1.9	31.0 ± 1.4
4	4	RTA/CFA	—	NRS	3.8 ± 0.1	24.0 ± 0.6	34.4 ± 2.9
5	4	RTA/CFA	—	Anti-IdX	1.6 ± 0.3	23.5 ± 1.2	33.0 ± 4.1
6	3	CFA	—	—	0.0 ± 0.0	3.8 ± 0.3	31.7 ± 1.8

* Rabbit serum was administered intravenously as 0.5 ml of neat serum; NRS, normal rabbit serum. All animals were killed on day 27, except two animals from group I that were killed at 14 d.

‡ Pathology scores were determined by examining hematoxylin and eosin–stained sections and assigning a score on a scale of 0 to 4 +: 0, no interstitial infiltrate; 1 +, <25% of cortex involved with interstitial infiltrate; 2 +, 25–50%; 3 +, 50–75%; 4 +, >75%.

§ DTH was measured with an engineer's micrometer as incremental footpad swelling in 10^{-3} in compared with control.

I This group comprised two animals from group I that were killed at day 14 of the experiment to establish midpoint renal pathology scores.
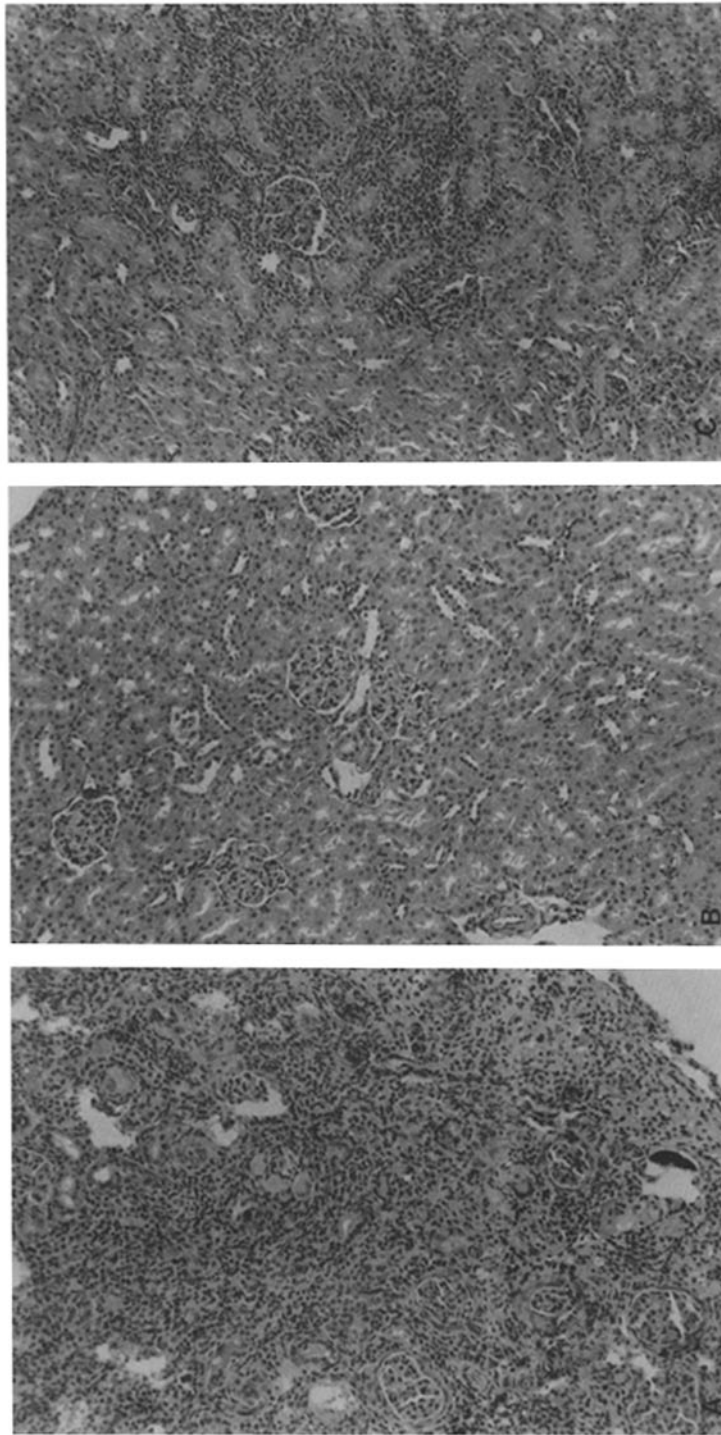
FIGURE 7. Representative pathology from studies using anti-IdX as a therapeutic modality. (A) Kidney section from group 2 animal. There is an intense interstitial infiltrate with obliteration much of the tubulointerstitial architecture. Hematoxylin and eosin, ×125. (B) Kidney section from group 3 animal. There is an absence of infiltrating cells and the tubulointerstitium is otherwise normal appearing. Hematoxylin and eosin, ×125. (C) Kidney section from group 5 animal. A patchy interstitial infiltrate is evident. Hematoxylin and eosin, ×125.
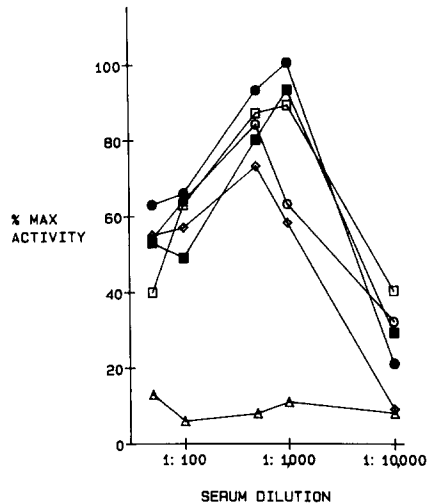
FIGURE 8. Serological anti-TBM-Ab response in animals from anti-IdX experiment. In a solid-phase RIA, wells were lined with 3M-1 (250 ng/well) and were reacted with serial dilutions of pooled sera from each treatment group. The reaction was developed with ^{125}I–anti–rat IgG. All RTA/CFA-immunized groups made substantial titers of anti-3M-1 antibodies. (—●—) group I; (—■—) group II; (—◇—) group III; (—○—) group IV; (—□—) group V; (—△—) group VI. Maximum cpm in this assay was 16,856.

effective in preventing the development of interstitial injury and in halting its progression. Representative interstitial lesions are shown in Fig. 7. DTH reactions to SRTA were similar among all groups except groups 3 and 6, which had greatly reduced responses (Table III). Periimmunization treatment of group 3 with anti-IdX-Ab, therefore, resulted in the abrogation of T cell responses producing both DTH (24) and the development of interstitial lesions. Group 5 rats treated with anti-IdX-Ab 2 wk after immunization with RTA/CFA experienced an arrest in the progression of disease, but no loss of DTH reactivity, which is consistent with presence of residual interstitial infiltrates and some antigen-reactive T cells. Animals in all groups made excellent and indistinguishable DTH responses to PPD, and all groups immunized with RTA/CFA produced comparable anti-TBM-Ab (anti-3M-1) responses (Fig. 8).

Discussion

A detailed analysis of autoreactive responses may not only reveal critical features of immunopathogenesis, but also may suggest potential therapeutic strategies for interrupting destructive autoimmune events. In the current study, 22 anti-TBM mAbs from five different fusions were isolated and characterized for just such an analysis. Because antibodies with specificity for the TBM do not arise spontaneously in BN rats, lymphoid cells were obtained from animals immunized with a xenogeneic tubular antigen, which is the standard method for inducing experimental anti-TBM disease. All monoclonals identified as preliminary positives by immunofluorescent criteria (i.e., staining of BN rat TBM, but not Lewis rat TBM; the latter strain lacks the target antigen of anti-TBM disease) were uniformly reactive with the nephritogenic antigen of anti-TBM disease, 3M-1. This suggests that the autoreactive B cell response in BN rats is quite restricted in its specificity to that family of basement membrane constituents bearing 3M-1 epitopes. This is consistent with the finding that polyclonal antisera from animals injected to produce disease stain only TBM-related structures (3).

Quantitative assessment of the TBM-reactive B cell repertoire, based on our fusion experience, indicates the presence of ~58 distinct clones. This estimate is based on the identical pair formula as modified by Briles and Carroll (18), and depends on the finding that some of the 22 anti-TBM mAbs were identical by criteria of subclass, light chain, relative affinity, and isoelectric focusing pattern. Because our fusions used lymphoid cells of antigen-primed and -boosted animals, it is possible that the naive TBM-reactive premature B cell population is somewhat larger than the above estimate. This is because the selective pressure of immunization may tend to favor expansion of those B cells bearing surface Ig of somewhat higher affinity. A similar approach has been used to quantify the anti-DNA B cell repertoire in the autoimmune (NZB × NZW)F$_1$ mouse at 80 distinct clonotypes (25). Other estimates in mice provide figures that range from 100 to 300 for antistreptococcal group A carbohydrate (anti-GAC) antibodies (26) to several thousand for anti-4-hydroxy-5-iodo-3-nitrophenyl acetyl (anti-NIP) antibodies (27).

The molecular basis of the anti-TBM B cell repertoire was analyzed using probes that define distinctive murine V$_H$ region gene families and crosshybridize with rat V$_H$ genes families (22, 28). Comparable data in the rat, to our knowledge, does not exist for other autoimmune B cell repertoires. Dominant V$_H$ gene family representation amongst murine autoantibodies, however, has been reported in several studies (29–32). These mouse autoantibodies, while varying in their specificities, nevertheless, can sometimes display preferential use of the 3' families, Q52 and 7183, although this is not always the case (33). They also frequently possess crossreactive idiotypes not always restricted to single a V$_H$ gene family (31, 34). This work in the mouse is surprisingly consistent with our data regarding both V$_H$ gene family use by anti-TBM mAbs and the expression of IdX. Four of six of our antibodies were derived from Q52- and 7183-like genes (Table II). These data represent the first suggestion that biased usage of V$_H$ gene families for autoantibody generation in the mouse might similarily apply to the rat. Three of our IdX-bearing anti-TBM mAbs (56R-3, 56R-7, and 57R-2), which were examined for heavy chain gene origin, crosshybridized with either Q52 or 7183 probes (Table II; Fig. 4). IdX$^-$ mAb (57R-1), which was also subjected to V$_H$ gene analysis, was derived from neither Q52 nor 7183 families. Our findings are consistent with a biased V$_H$ gene usage among rat autoantibodies, that a crossreactive idiotype is readily demonstrable, and that such an IdX can be specified by more than one V$_H$ gene family. Although some IdX antibodies can also be specified by V$_L$ genes (35), we have observed, in preliminary experiments, that our anti-IdX-Ab only binds to heavy chains on Western blots (data not shown). Additionally notable is the finding that both 56R-7 and 57R-2 share the same V$_H$ gene family (Q52) but have substantially different affinity constants (6 × 10^6/mol and 2 × 10^8/mol, respectively). This may reflect contributions to binding affinity made by either relatively small differences in V$_H$ chain amino acid sequence, light chain differences, or D or J chain differences.

On the surface it would also seem from our quantitative estimate of B cell clonotypes and the eclectic use of V$_H$ gene families that the B cell repertoire producing anti-TBM-Ab would be at least moderately diverse. Closer analysis,

however, reveals striking similarities among these otherwise distinctive monoclonals. Virtually all the anti-TBM-Ab competitively inhibit the recognition of 3M-1 by a radiolabeled reference anti-TBM mAb, suggesting the common recognition of the same or nearly identical epitope. This point is qualified by the consideration that some inhibition could be secondary to stearic hindrance or conformational changes in 3M-1 after anti-TBM-Ab binding to an unrelated epitope, and must await further peptide analysis. A single anti-TBM mAb (56R-9) is also capable of inhibiting >80% of the binding of polyclonal human anti-TBM-Ab antisera to its ligand, human 3M-1 (Fig. 6). Taken together, these findings support the concept that while the humoral immune response in rats and humans with anti-TBM disease is formed by a moderately diverse population of B cells, the paratypic specificity of this response is focused to an immunodominant epitope, and conserved among some mammals.

This conserved paratypic specificity in rats is also reflected in the demonstration of broadly crossreactive idiotype. Crossreactive idiotypes have previously been described in experimental thyroiditis (36, 37) and in lupus mice (25). Although the induction of antiidiotypic immunity with anti-IdX-Ab was partially effective in attenuating progressive renal injury in lupus mice (38), our anti-IdX antisera, when administered to BN rats at the time they were immunized to produce anti-TBM disease, was capable of specifically preventing renal injury. These animals also had attenuated T cell responses (as measured by DTH to SRTA), but only very slightly lower serum anti-TBM-Ab titers. The ability of anti-IdX antisera to influence T cell responses may reflect the previously documented serologic sharing of idiotypic determinants between anti-TBM-Ab and 3M-1-specific T cells (4). That such treatment did not markedly influence the amplitude of the anti-3M-1 antibody response is consistent with results of xenogeneic antiidiotypic manipulation in other systems (39, 40). Further, the finding that humoral immune responses to 3M-1 were similar among all RTA/CFA immunized groups (Fig. 8), implies that anti-TBM-Ab, per se, in a uniformly susceptible strain, are not sufficient for the induction of disease (3). Also of note was the demonstration that anti-IdX antisera was effective in arresting the progression of disease when given 2 wk after immunization at a time when moderately severe interstitial nephritis was already present. While previous studies from this laboratory (24, 41) and others (42) have documented the effectiveness of antiidiotypic manipulation in anti-TBM disease, this is the first demonstration of the therapeutic usefulness of an anti-IdX-Ab even after effector T cell mechanisms had inflicted a moderate degree of renal damage. The available data do not provide a definitive statement regarding the lasting effect of this intervention. Thus, it is conceivable that kidneys of group 5 animals would have either progressed to severe interstitial damage or returned to normal over a time frame more prolonged than that used in this experiment. The resolution of this issue will require further study.

Summary

Experimental anti–tubular basement membrane (anti-TBM) disease is an autoimmune interstitial nephritis elicited in susceptible rodents after immunization with renal tubular antigen. The nephritogenic antigen in the immunizing

preparation is 3M-1, a 48,000 M_r noncollagenous glycoprotein. The hallmarks of the renal lesion are the presence of anti-TBM antibodies (anti-TBM-Ab) and a dense mononuclear cell infiltrate. The anti-TBM B cell repertoire in this disease was analyzed using a library of 22 anti-TBM mAbs generated in a prototypically susceptible Brown Norway rat. These anti-TBM mAbs were all demonstrated to be 3M-1 specific and their characterization formed the basis for the following observations: (a) The size of the anti-TBM B cell population is estimated at 58 distinct clones; (b) by competitive inhibition criteria, all anti-TBM mAbs recognize the same (or spatially close) epitope(s) on 3M-1. This focused recognition was maintained in spite of considerable variability in affinity. Epitopic dominance could also be demonstrated in human polyclonal anti-TBM antisera from a patient with anti-TBM disease; and (c) a crossreactive idiotype was documented, and antisera directed toward this set of variable region determinants was shown to be effective as a prophylactic regimen to abrogate disease, and as a therapeutic modality to arrest the progression of disease; (d) analysis of V_H gene families suggested biased usage of Q52- and 7183-like families, although at least three gene families are used in the anti-TBM-Ab response. Thus, the anti-TBM B cell compartment in BN rats is moderately large, but is primarily focused to a single epitope on the nephritogenic antigen and is associated with a disease-modifying crossreactive idiotype.

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