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Enrichment of Anti-Glomerular Antigen Antibody-Producing Cells in the Kidneys of MRL/MpJ-Fas\textsuperscript{lpr} Mice\textsuperscript{1}

Hideharu Sekine, Hiroshi Watanabe, and Gary S. Gilkeson\textsuperscript{2}

Lupus nephritis is characterized by immune complex deposition and infiltration of inflammatory cells into the kidney including Ab-producing cells (AbPCs). Although AbPCs play a central role in the pathogenesis of immune complex glomerulonephritis in lupus, the specificity and pathogenic role of AbPCs infiltrating into the kidneys in lupus are poorly understood. To characterize AbPCs present in lupus kidneys, we isolated AbPCs from diseased MRL/MpJ-Fas\textsuperscript{lpr} (MRL/lpr) mouse kidneys. ELISPOT assays, using glomerular Ag (GA) extracts as Ag, demonstrated significant enhancement of anti-GA AbPCs in the kidneys as compared in peripheral blood or spleen of the same mouse. We isolated hybridomas with anti-GA specificity from MRL/lpr mouse kidneys. All the anti-GA mAbs had polyclonal binding to ssDNA, dsDNA, and IgG (i.e., rheumatoid factor), but not to histones or Sm. Sequence analysis of anti-GA Abs suggested the occurrence of somatic mutations and amino acid replacement in complementarity-determining regions with a high replacement to silent ratio resulting in charged amino acids. Intravenous administration of the monoclonal anti-GA Abs into BALB/c mice resulted in graded deposition in glomeruli paralleling their ELISA anti-GA reactivity. These results suggest that AbPCs infiltrating the kidneys in MRL/lpr mice accumulate as a result of Ag selection and likely play a pathologic role in lupus nephritis.

arose as a result of Ag selection. These results suggest specificity and likely pathogenicity of renal infiltrating AbPCs in lupus nephritis indicated by the enrichment of anti-GA AbPCs compared with the spleen and peripheral blood.

Materials and Methods

**Mice**

MRL/MP-Fas<sup>−/−</sup> (MRL/lpr) mice, C3H/lpr (C3H/lpr), and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the Strom Thurmond Biomedical Research Building at the Medical University of South Carolina (Charleston, SC) under specific pathogen-free conditions.

**Isolation of mononuclear cells from MRL/lpr mouse blood, spleen, and kidney**

Spleens or kidneys were removed from 20-wk-old female MRL/lpr mice, then transferred into 5 ml of RPMI 1640 (Life Technologies, Rockville, MD) individually. Kidneys were finely minced with a sterile razor blade into 0.5-mm fragments and incubated with 1.0 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 for 15 min at 37°C. After the enzymatic digestion, both spleen and kidney fragments were mashed between frost-ended of two sterilized slides; then the cell suspension was washed (150 × g, 4 min) with RPMI 1640 and filtered first through a 70-μm cell strainer (BD Labware, Franklin Lakes, NJ) and then through a 40-μm cell strainer to remove large particles and large cells. The crude splenic or renal mononuclear cell suspensions were brought up to 10 ml by adding RPMI 1640 and mononuclear cells further purified by the Percoll (Amersham Pharmacal Biotech, Piscataway, NJ) density gradient method. Blood was obtained by retro-orbital bleeding and cardiac puncture, and collected into sodium-heparin tubes (BD Labware). Blood samples were also brought up to 10 ml by adding RPMI 1640 and mononuclear cell purification performed as done for spleen and kidney samples.

**Percoll density gradient centrifugation**

Percoll (Amersham Pharmacal Biotech) was made iso-osmotic by adding 1 part at 10× PBS to 9 parts of Percoll. Further dilution of the 90% Percoll was made with 1× PBS (for 20% Percoll) or 1× RPMI 1640 (for 60% Percoll) to make it easy to see the interface of 20% and 60% Percoll. Centrifugation was performed using 50 ml polycarbonate tube (Nalg Nunc International, Rochester, NY). The spleen, kidney, or blood cell suspension prepared as previously described was carefully layered onto 12 ml Nunc International, Rochester, NY). The spleen, kidney, or blood cell suspension prepared as previously described was carefully layered onto 12 ml Nunc International, Rochester, NY) of 20% Percoll on a cushion (10 ml) of 60% Percoll by a Pasteur’s glass pipette. The samples were then centrifuged for 5 min at 15,000 × g at 8°C with brakes off in a fixed angle JA-20 rotor (Beckman Coulter, Fullerton, CA). Mononuclear cells were collected from the 20% and 60% interface and washed with more than 10× volume of Dulbecco’s PBS (Life Technologies).

**ELISPOT**

ELISPOT assays were used to assess the number of anti-GA AbPCs in spleen cells, PBMCs, and kidney mononuclear cells. The 96-well microtiter plates (Millipore, Bedford, MA) coated overnight with 50 μl/well 50 μg/ml rat GA solution in PBS were washed with endotoxin-free Dulbecco’s PBS containing 0.25% Tween 20 and blocked with 200 μl/well PBS containing 3% BSA for 1 h at 37°C. The plates were washed and rinsed with DMEM containing 3% BSA and incubated in duplicate with splenocytes, PBMCs, or kidney mononuclear cells in serial dilutions starting at 0.5–1 × 10<sup>5</sup> cells/well in 100 μl of DMEM containing 3% BSA, 2.5% PBS, streptomycin (100 μg/ml), and penicillin (100 U/ml). Following an 18-h incubation at 37°C in 5% CO<sub>2</sub>, the plates were washed six times with PBS containing 0.25% Tween 20. The plates were then incubated for 2 h at 37°C with 50 μl/well of 1 μg/ml biotinylated goat anti-mouse IgG Ab (Southern Biotechnology Associates, Birmingham, AL), washed six times with PBS containing 0.25% Tween 20, and incubated for 2 h at room temperature with 50 μl/well of 1 μg/ml streptavidin-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in PBS containing 0.25% Tween 20. Following washing six times with PBS containing 0.25% Tween 20 and once with PBS, the plates were developed with 50 μl/well of a substrate solution containing 3-amino-9-ethylcarbazole (Sigma-Aldrich; 2.5 mg dissolved in 200 μl of 0.1% dimethylformamide) in 9 ml of 0.05 M sodium acetate, pH 5.0, and 4 μl of 30% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich). The process was stopped by washing with tap water, and

**Hybridomas**

After washing and counting, renal mononuclear cells derived from three mice as previously described were added 5:1 to the fusion partner SP-2 cells in 50% polyethylene glycol (Boehringer Mannheim, Indianapolis, IN). After washing, cells were suspended in hyponxanthine/aminopterin/thymidine selective medium containing 10% PBS and penicillin-streptomycin. Cells were then plated at 2 × 10<sup>5</sup> cells per well, and supernatants were screened for rat GA binding by ELISA. Cells from wells without anti-GA Ab were cloned twice by limiting dilution. Ab isotypes were determined by ELISA using goat anti-mouse Ig subclass specific Abs (Southern Biotechnology Associates). Abs from supernatants were purified by affinity chromatography using protein A-Sepharose or goat anti-mouse IgM sepharose columns (Bio-Rad, Richmond, CA), followed by dialysis against PBS. Concentrations of Abs were determined by spectrophotometric measurement at 280 nm.

**GA preparation**

GA was isolated from glomeruli according to a variant of the published methodology (26). Briefly, glomeruli were harvested from PBS-perfused kidneys of Lewis rats by using metallic mesh. The insoluble pellet was washed four times in cold double-deionized water (4°C). The final pellet was lyophilized and stored at −80°C. Before use for ELISA or ELISPOT, lyophilized GA was weighed, resuspended with PBS and, sonicated at the appropriate concentration for each experiment.

**ELISA**

Ab binding to GA, DNA, IgG (i.e., RF), total histones and Sm was assessed by ELISA. Anti-GA ELISA was performed as previously described with some modifications (27). GA was suspended in PBS at a concentration of 80 g/ml and coated at 50 μg/ml on 96-well polystrene ELISA plates (Dynatech Laboratories, Chantilly, VA). The plates were incubated for 90 min at room temperature, washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 60 min with PBS-T containing 1% BSA (PBS-T; Sigma-Aldrich). After washing, the plates were treated with 50 μl/well DNase I (Sigma-Aldrich) diluted 50 U/ml in PBS containing 5 mM MgSO<sub>4</sub> for 3 h at 37°C. The DNase-treated plates were then washed, and mAbs (starting concentration of 10 μg/ml or sera (starting dilution of 1/100) diluted in PBS-T were transferred to the plates and incubated for 90 min at room temperature. The plates were then washed, and HRP-conjugated goat anti-mouse IgG (γ-chain-specific, Sigma-Aldrich) was added at a dilution of 1:4000. For IgM mAb (clone CS-12), HRP-conjugated goat anti-mouse IgM (μ-chain-specific; Sigma-Aldrich) was added at the same dilution. Following a 45-min incubation, the plates were washed and substrate solution containing 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was added in 0.1 M citrate buffer, pH 4.0, and 0.015% H<sub>2</sub>O<sub>2</sub>. After incubation for 30 min, plates were read on a Fluoroscan plate reader at OD<sub>405</sub> (Dynatech Laboratories). Serum pooled from BALB/c mice was used as a negative control and serum pooled from 20-wk-old MRL/lpr mice was used as a positive control. Additional controls included isotype-matched Ig for each of the mAbs.

The anti-DNA ELISA was performed as previously described (28). Briefly, ELISA plates were coated with 5 μg/ml calf thymus dsDNA (Sigma-Aldrich) at 37°C overnight. The plates were then washed with PBS-T and blocked for 60 min with PBS-T. mAbs or sera diluted in PBS-T were transferred to the plates and incubated for 45 min at room temperature. The assay was then performed as previously described with HRP-conjugated goat anti-mouse IgG or goat anti-mouse IgM.

RF reactivity was measured as previously described with minor modifications (25). ELISA plates were coated with 1 μg/ml rabbit IgG (Sigma-Aldrich) overnight at 4°C. For anti-mouse IgG RF ELISA, mouse IgG2a or IgG3 (Southern Biotechnology Associates) were used for coating at same concentration (1 μg/ml). After washing and blocking, mAbs or control sera diluted in PBS-T were transferred to the plates and incubated for 90 min at room temperature. After incubation, the plates were washed and HRP-conjugated rabbit anti-mouse IgG (Fc-specific; Pierce, Rockford, IL) or rabbit anti-mouse IgM (μ-chain-specific; Pierce) was added at a dilution of 1/4000. For anti-mouse IgG RF ELISA, HRP-conjugated goat anti-mouse IgG1, IgG2b, or IgM (Southern Biotechnology Associates) was added at a dilution of 1/4000. The assay was then developed as previously described.

For the anti-histone ELISA, ELISA plates were coated with 10 μg/ml calf thymus histone (natural mixture of the histones H1, H2A, H2B, and H4; Boehringer Mannheim) in 0.05 M carbonate buffer (pH 9.5), washed, and blocked with 1% BSA. After washing, mAbs or control sera diluted in PBS-T were transferred to the plates and incubated for 90 min at room temperature. After incubation, the plates were washed and HRP-conjugated goat anti-mouse IgG1, IgG2b, or IgM (Southern Biotechnology Associates) was added at a dilution of 1/4000. The assay was then developed as previously described.
9.6), and incubated at 4°C overnight. After washing and blocking with PBS-TB for 1 h, mAbs or sera diluted in PBS-TB were transferred to the plates and incubated for 2 h at room temperature. The assay was then performed as previously described with HRP-conjugated goat anti-mouse IgG or goat anti-mouse IgM.

The anti-Sm ELISA was performed as previously described (29). Briefly, ELISA plates were coated with 10 μg/ml Sm Ag (Immunovision, Springfield, AR) in borate-buffered saline (BBS) at 4°C overnight, washed, and blocked with BBS containing 0.05% Tween 20 and 1% BSA. All washes were done with BBS containing 0.05% Tween 20. mAbs or sera diluted in BBS containing 0.05% Tween 20 and 1% BSA were transferred to the plates and incubated for 2 h at 4°C. The assay was then performed as earlier described with HRP-conjugated goat anti-mouse IgG or goat anti-mouse IgM except for using BBS instead of PBS.

**Immunoblotting**

Binding of anti-GA Abs to glomerular protein Ags was verified by Western blotting. GA (20 μg) was electrophoresed on 4–20% linear gradient SDS-polyacrylamide gels (Bio-Rad) and electropho-botted onto a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with primary anti-GA Abs or isotype-matched control mouse Abs at 10 μg/ml diluted in TBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dried milk. After washing, membranes were incubated with AP-conjugated goat anti-mouse Ig (Southern Biotechnology Associates) and processed for ECF detection (Amersham, Arlington Heights, IL). Additional blots were done after DNA analysis of both the GA preparation and the mAbs.

**RNA sequencing**

Total RNA was isolated from hybridoma lysates using TRIZol reagent (Life Technologies). Poly(A) plus RNA was then isolated from total RNA using oligo(dT) cellulose columns (Life Sciences). RNA sequencing was performed by direct primer extension reaction using the dideoxynucleotide method with avian myeloblastosis virus reverse transcriptase (Life Sciences, Ft. Lauderdale, FL). For each reaction, 5–10 μg mRNA was primed with 40 ng of [32P]ATP-labeled synthetic oligonucleotide primer. The oligonucleotide primers used were: constant region, (κ) 5′-TGGGAT GGTTGGGAAGATG-3′, (γ) 5′-GGGGCCAGTGATATGAC-3′, and (μ) 5′-GCAGGAGACGGGGGG-3′; Jκ regions (Jκ1) 5′-CCAGCTTTGT GCCCTCA-3′, (Jκ2) 5′-CCAGCTTTGGCTCCACT-3′, and (Jκ5) 5′-AT CAGCCCCATTAC-3′; and Vμ framework 5558 FR Sequence reaction products were electrophoresed on 6–10% polyacrylamide (Long Ranger Gel solution; AT Biochem, Malvern, PA) 7 M urea gels. Gels were dried and exposed to Amershaw Hyperfilm MP (Amersham Pharmacia Biotech) or Kodak Biomax MS film (Rochester, NY). Each V region exon was sequenced two or more times.

**Database search**

V region sequences encoding anti-GA Abs from MRL/lpr mice were compared with murine sequences in GenBank database (www.ncbi.nlm.nih.gov/igblast/) using the fasta program. They were compared with murine sequences in GenBank database (www.ncbi.nlm.nih.gov/igblast/) using the fasta program.

**Administration of purified mAbs to normal mice**

A total of 1.0 mg of purified anti-GA mAbs (E5-1, H3-9, and G1-12) or isotype-matched control Abs (IgG1, IgG2b; Sigma-Aldrich) or were injected into the tail vein of 8- to 10-wk-old female BALB/c mice. C3H-F/Fs, a monoclonal anti-dsDNA Ab derived from a C3H/lpr mouse was used as an additional control (28). C3H-Fs/Fs has no anti-GA activity by ELISA. After 3 h, the animals were sacrificed and the kidneys were removed and snap frozen in liquid nitrogen. Frozen kidneys were then placed in OCT medium and sliced sections (4-μm thick) were stained with fluorescein-conjugated goat anti-mouse IgG (Cappel, Durham, NC).

**Statistics**

Data are summarized as the mean ± SEM. To calculate significance levels between groups, the Wilcoxon rank-sum test was used. Differences between values were considered significant at p < 0.05.

**Results**

**ELISPOT**

To determine whether anti-GA AbPCs were present in MRL/lpr mouse kidneys, we used the anti-GA ELISPOT assay. Twenty-week-old female MRL/lpr mice were sacrificed as well as age-

**Isotype and binding activity of anti-GA Abs**

To analyze the binding and molecular properties of Abs produced by individual anti-GA AbPCs infiltrating the kidneys of MRL/lpr mice, we generated mAbs from AbPCs derived from MRL/lpr mouse kidneys. To gain enough cells for hybridoma production, mononuclear cells from the kidneys of three 20-wk-old MRL/lpr mice were combined. Following standard fusion procedures, the wells demonstrating cell growth were screened for anti-dsDNA

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**FIGURE 1.** Frequency of anti-GA AbPCs in kidneys, peripheral blood, and spleens of MRL/lpr mice. Frequency of anti-GA AbPCs in kidneys, peripheral blood, or spleens of 20-wk-old female MRL/lpr mice were measured by ELISPOT. Age- and sex-matched splenocytes of C3H/lpr and BALB/c mice were used for control experiments. To present the data as the relative number of anti-GA AbPCs in the total B cell number in each sample, the populations of CD19+ cells (i.e., B cells) in each sample were analyzed by flow cytometry at the same time. Data presented are the mean ± SEM. Confidence intervals were calculated using Wilcoxon signed ranks test. *, p = 0.043; #, p = 0.027.
and anti-GA activity. All wells with anti-GA activity had anti-dsDNA activity, however not all wells with anti-dsDNA activity had anti-GA activity (~50%). Four anti-GA hybridomas were derived for further study. Of the four mAbs studied, three were IgG (two were IgG1, one was IgG2b) and one was IgM (Table I).

Binding activities of the mAbs to GA, ssDNA, dsDNA, rabbit total IgG, mouse IgG2a, mouse IgG3, whole histones, and Sm were measured by ELISA. As shown in Fig. 2, all the mAbs had multiple binding activities. H-3 and G-12 showed not only high binding to GA, but also had strong binding activities to DNA and IgG, respectively. We also measured reactivity to other protein Ags, however, none of the mAbs showed binding activity to either whole histones or Sm preparations. Isotype-matched control mAbs did not have binding activity above background for any Ag (data not shown). Binding of pooled BALB/c and MRL/lpr sera are provided in Fig. 2 as positive and negative controls using an anti-IgG secondary Ab. Similar assays were run using an anti-IgM secondary Ab. BALB/c pooled sera had no IgM reactivity above baseline for any Ag. The MRL/lpr pooled sera had IgM reactivity above an OD340 of 0.1 only for dsDNA (0.392), rabbit IgG (0.538), and mouse IgG2a (0.334).

We verified binding activities of the four mAbs to protein GAs by the immunoblotting method. Fig. 3 shows the SDS-PAGE analysis of the GA preparation and Western blotting with the four mAbs under reducing conditions. In Fig. 3A, the GA sample appeared as one main band with a broad smear of other proteins. In contrast to the results of SDS-PAGE analysis, Western blotting with the four mAbs detected several bands bound within the smear. The molecular size of one band (33 kDa) was identical with the band that was visible by SDS-PAGE analysis. Another band of 37 kDa is observable in the blots of all four Abs. Other bands are variably bound by the different Abs. DNAsing the glomerular extract and the Ab preparations did not affect the binding by Western blotting of the mAbs (data not shown). Thus, these results indicate that the renal-derived mAbs bind to glomerular protein Ags directly and do not merely bind to DNA alone within the glomerular extract.

H and L chain V region usage of anti-glomerular Ag Abs

The Ab variable region nucleotide sequences, determined by direct sequencing of hybridoma mRNA, are available from GenBank database under accession numbers AY081855 to AY081862. By comparison with sequences recorded in GenBank, three of the Ab H chains used J558 VH genes, whereas one used a Q52 VH gene (Table I). Although three hybridomas used J558 family genes, none of the hybridomas were clonally related, because they differed in DH and JH gene usage (Table I). VH and Jκ gene usage among the hybridomas was diverse (Table I) and showed no significant bias for a specific gene segment.

CDR sequences of anti-glomerular Ag Abs

The CDR sequences were analyzed for sequence motifs considered important for DNA and GA binding. Previous analyses have demonstrated that VH CDR3 arginines promote DNA binding through charge-charge interactions with the phosphate backbone as well as hydrogen bonding with guanine bases via the major and minor grooves (30, 31). Arginine content is significantly increased in VH CDR3, Vκ CDR1, and Vκ CDR3 of spontaneous anti-DNA from lupus mice compared with non-DNA binding Abs (32, 33). High content of arginine is also observed in VH CDR3 of glomerular binding mAbs (21.4%) derived from spleen cells of MRL/lpr mice (34). As shown in Fig. 4, arginines were present in Vκ CDR1 and Vκ CDR2 of three of four anti-GA mAbs. Although all anti-GA mAbs have binding activity to ssDNA and dsDNA, none of the Abs had arginines in their VH CDR3. Other charged amino acids are present within the VH CDR3, however, possibly contributing to GA binding activity.

Somatic mutations

The occurrence of somatic mutations in Ig V regions contributes to the conventional Ag-driven response (32, 35–37). To assess whether anti-GA AbPCs in MRL/lpr mouse kidneys were derived from Ag-driven responses, we compared anti-GA Ab V region nucleotide sequences with germline gene sequences identified in a GenBank search. Fig. 5 shows Vκ sequence similarity between the anti-GA mAbs and reported germline mAbs. As shown in Fig. 5A, the Vκ E5-1 differed from the germline gene Vκ 21–10 by four nucleotides, all of which involved an amino acid acid replacement. Three of four involved a change to positively charged amino acids, arginine or lysine. Similar analysis of the Vκ H3-9 revealed five nucleotide differences from the germline gene Vκ cp9, three of which involved changes to arginine and aspartic acid, negatively or positively charged, respectively, in CDR1, and one of which involved the insertion of an amino acid acid replacement. Of the four involved a change to positively charged amino acids.

At this time, we cannot verify that Vκ 21–10, Vκ cp9, Vκ 12–41, and Vκ 19–15 are the germline genes for Vκ 21–10, Vκ cp9, Vκ 12–41, and Vκ 19–15 are the germline genes for Vκ E5-1, Vκ H3-9, Vκ G1-12 and Vκ C5-12, respectively. However, the findings of 10 high replacement to silent ratio (≥2.0), 2 frequent occurrence of amino acid replacement within CDRs, and 3 replacement primarily to charged amino acids suggests these somatic mutations occurred as a result of Ag selection and did not occur randomly.

We also performed a homology search of the four mAbs for the closest reported germline VH gene. However, the gene search revealed there were no sequence similarities of ≥95% between mAbs VH and germline genes VH. Thus, we cannot be certain as to the VH germline gene of origin for these four Abs.

In vivo evaluation of anti-GA mAbs

To assess the potential pathogenic relevance of anti-GA reactivity, anti-GA IgG mAbs (E5-1, H3-9, and G1-12) were i.v. injected into BALB/c mice. For comparison, IgG1 and IgG2b isotype-matched mouse IgGs were used as controls. After administration of anti-GA mAbs to normal mice, the kidneys of all mice revealed diffuse deposits of IgG in glomeruli in vivo (Fig. 6). The staining of the glomeruli involved both the mesangial areas as well as the capillary loops in a granular staining pattern consistent with IC deposition. The fluorescent intensity of IgG deposition in the kidneys correlated with the results of anti-GA binding activity measured by ELISA (τ = 1.0, when comparing the OD340 of anti-GA ELISA
with the grade of fluorescent intensity by calculating with Spearman’s correlation coefficient by rank). By comparison, none of the mice receiving isotype-matched control Igs (i.e., IgG1 and IgG2b) or an IgG2a anti-DNA Ab without anti-GA activity from a C3H/lpr mouse had IgG deposition in their glomeruli (data of control IgG2b and C3H/lpr IgG2a injection are not shown). These results revealed the strong binding activity of anti-GA IgG mAbs to glomeruli in vivo.

Discussion

The results presented in this study provide new insight into the specificity and selection of in situ renal AbPCs in murine lupus nephritis. Although B cells and plasma cells are known to infiltrate the kidneys of humans with SLE as well as animal models of the disease, whether they had specificity for self Ags in the kidney was previously unknown (2, 3). By finding enrichment of GA-specific AbPCs in the kidney, our results suggest that there is selective recruitment and/or retention of AbPCs with reactivity for local Ags in the lupus kidney. Indeed, we found that up to 10% of AbPCs in the kidneys of MRL/lpr mice are anti-GA AbPCs. In addition, the mAbs isolated were found to have limited polyreactivity. Molecular analysis of the Abs indicated they were somatically mutated with characteristics of Ag selection.

We did not find evidence for renal germinal center formation as staining for peanut agglutinin in the kidneys of diseased MRL/lpr mice was negative (data not shown). Thus, we believe that these autoreactive AbPCs somatically mutated and clonally expanded in the spleen or lymph nodes before migration to the kidney. MRL/lpr B cells can, however, somatically mutate and receptor edit outside of germinal centers in marginal zones (38). Thus it is possible, although we believe unlikely, these anti-GA AbPCs are proliferating and mutating in the kidney without germinal center formation. Definitively determining if the kidney is “immunologically active” would require extensive further experimentation beyond the scope of the current studies. Regardless of where their maturation and expansion occurred, it is clear anti-GA AbPCs are present in the lupus kidney and the significant enrichment of this cell population suggests they are selectively recruited and/or retained in the kidney and are likely playing a role in disease.

Administration of the mAbs derived from renal AbPCs to normal mice resulted in glomerular binding in vivo confirming the nephrophilic properties of Abs produced by renal infiltrating AbPCs. Although the production of glomerular binding autoantibodies contributes to the pathogenicity of these cells in lupus nephritis, it is unlikely that it is the key pathogenic mechanism of these cells in lupus nephritis. Abs produced by these cells would still have to enter the circulation to be deposited as ICs in glomeruli. Thus local production would not be more pathogenic than Abs produced in lymph nodes or in the spleen. We believe it is more likely these cells contribute to disease via Ag presentation to autoreactive T cells and perhaps additionally through secretion of cytokines (39). Indeed, in some inflammatory responses, B cells are the primary source of cytokine production (40). The AbPCs serving as Ag presenters or cytokine producers is consistent with the model proposed by Chan et al. (41) regarding the role of the B cell in autoimmunity. Total elimination of B cells prevented disease in MRL/lpr mice. In contrast, in MRL/lpr mice in which B cells are present, but cannot secrete Ab, renal disease develops, albeit not as severe as in mice with intact B cell function (41). Thus, B cells

FIGURE 2. Reactivity to various Ags by anti-GA Abs. Reactivity to GA, ssDNA, dsDNA, rabbit total IgG, mouse IgG2a, mouse IgG3, histones, and Sm by anti-GA mAbs was measured by ELISA. mAbs and control sera were tested at 10 μg/ml or a 1/100 dilution, respectively. OD values were represented as mean values of triplicate measurement. Pooled MRL/lpr mice or BALB/c mice sera (20-wk-old, n = 4) were used for positive and negative controls. Data presented for the controls were generated using an anti-IgG secondary Ab.

FIGURE 3. Western blotting of GA with anti-GA mAbs. A, SDS-PAGE of GA (20 μg) under reducing conditions is shown. The proteins were stained with Coomassie blue. B, Western blotting of GA with anti-GA mAbs under reducing conditions. Es-1 (lane 1); H3-9 (lane 2); G1-12 (lane 3); C5-12 (lane 4); control mouse (lane 5) IgG1 mAb; control mouse (lane 6) IgG2b mAb; control mouse (lane 7) IgM mAb.
FIGURE 4. $V_H$ and $V_K$ CDR amino acid sequences of anti-GA mAbs. A, $V_H$ CDR amino acid sequences of anti-GA mAbs are shown. Charged amino acids (D, E, K, R, and H) and asparagines (N) are underlined. Numbering of amino acid positions is as suggested by Kabat et al. (35). Gaps in $R,$ and $H$) and asparagines (N) are underlined. Numbering of amino acid positions is as suggested by Kabat et al. (35). Gaps in $V_H$ CDR sequences were introduced to align sequences for proper numbering.

<table>
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<tr>
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clearly play an important role in autoimmunity above and beyond the secretion of autoantibodies. We propose these additional functions are performed by anti-GA AbPCs in the lupus kidney and explain the enrichment of these cells in the lupus kidney.

Interstitial infiltrates of mononuclear cells, including B cells and plasma cells are seen in the majority of cases of human and animal models of lupus nephritis (4, 16–19). In general, the degree of infiltration is proportional to the severity of the glomerular lesion (16). Initiation of lupus nephritis is thought to result largely from deposition of IC in the glomerular capillary wall (4). IC deposition is observed in the early stages of disease and in most models before mononuclear cell influx. Previously, it has been unclear whether in situ renal AbPCs were present due to autoreactive specificity for renal Ag(s) or were randomly attracted to chemotactic factor(s) such as B lymphocyte chemokine (CXC chemokine ligand 13) (42). Other factors derived from the complement activation cascade or mesangial cells initiated by glomerular IC deposition may also attract B cells to the kidney (43). A recent study reported significant numbers of plasmablasts in the kidneys of NZB/NZW mice and found that anti-OVA AbPCs induced by OVA immunization were present in the kidneys of diseased mice. These investigators suggested, based on these findings, that the renal AbPCs in lupus were drawn to the kidney on a nonspecific basis due to the ongoing inflammatory response. There was no data presented, however, on the presence of autoreactive AbPCs, the specificity of the non-anti-OVA AbPCs present in the kidneys of NZB/NZW mice, or the relative frequency of anti-OVA AbPCs in the kidney compared with the spleen (44). If the mechanism of AbPCs recruitment and retention in the kidney was via chemokines alone, the AbPCs would be of random specificity (42). Such nonspecific recruitment would explain the presence of OVA reactive AbPCs in the kidneys of NZB/NZW mice following OVA immunization (44). Such nonspecific recruitment alone does not, however, explain the enrichment of anti-GA AbPCs in the kidneys of MRL/lpr mice.

Consistent with our findings of enrichment of renal Ag specific cells in the lupus kidney, analysis of TCR $V_\beta$ gene expression in the kidneys of human SLE demonstrated oligoclonal expansion compared with peripheral blood. The finding of oligoclonal expansion suggests these renal T cells were clonally proliferating in the kidney in response to local Ags (21). Our data suggest that a similar enhancement of AbPCs is occurring in the lupus kidney based on the specificity of these cells for glomerular Ags. We hypothesize that AbPCs initially nonspecifically infiltrate into the kidney drawn by chemokines. AbPCs with specificity for glomerular Ags preferentially remain in an Ag-exposed region, playing a pathogenic role by Ag presentation and/or cytokine secretion. The mechanism for this preferential retention in the kidney is unclear, but is likely similar to the enrichment of RF producing AbPCs in the synovium of patients with rheumatoid arthritis.

The specificity of Abs deposited in the kidneys of lupus patients and lupus mice was first assessed over 30 years ago and recently readdressed. These studies all indicate an enrichment of autoantibodies, especially anti-DNA Abs, in the renal eluates compared with serum. Eluted Ig from the kidneys of both human SLE patients and MRL/lpr mice is polyreactive with demonstrable binding to various autoantigens (e.g., DNA, DNA-histone complexes, laminin, and others) (10, 45, 46). Based on the consistent findings of all these studies, it appears that polyreactivity is a distinguishing feature of nephritogenic autoantibodies. Similar to the reported eluted nephritogenic autoantibodies, the anti-GA mAbs from kidney derived AbPCs demonstrated polyreactive binding to ssDNA, dsDNA, and IgG. The Abs, however, did not bind to histones alone or Sm similar to a recent Ab microarray analysis by Xie et al., of Abs eluted from murine lupus kidneys (47). Our data indicate that cells producing these pathogenic Abs are present in the kidney enriched compared with the spleen and peripheral blood.

The primary focus of most investigations of lupus nephritis is on the glomerular disease. Tubulointerstitial inflammation is also a key component of disease and renal AbPCs could be playing a role in lupus nephritis via initiating or enhancing interstitial disease. AbPCs are found in the interstitium of nephritic kidneys in human and murine lupus (22). Tubulointerstitial IC depositions are frequently observed in lupus nephritis (4, 16); in contrast to glomerular IC deposits, tubulointerstitial IC deposits may occur via local secretion of Ab by interstitial AbPCs. The target Ag of these interstitial Abs is unknown at present. MRL/lpr mice with functional B cells expressing surface Ig, but lacking Ab secretion, developed interstitial nephritis characterized by cellular infiltration similar to wild-type mice, whereas mice with no B cells developed no interstitial nephritis (41). These results suggest infiltrating B cells are essential to initiate interstitial inflammation either as APCs or as cytokine-producing cells. Ab production does not appear to be required for interstitial disease in lupus nephritis (41).

In conclusion, our results demonstrate that 1) AbPCs with specificity for GAs are markedly enriched in murine lupus kidneys
FIGURE 5. Occurrence of somatic mutations and amino acid replacement in anti-GA mAbs. V\(_{\kappa}\) sequence similarity between anti-GA Ab E5-1 and germ-line gene 21–20 (A), anti-GA Ab H3-9 and germ-line gene cp9 (B), and anti-GA Ab C5-12 and germ-line gene 19–15 (D). Dashes denote nucleotide identity. Where nucleotide sequence differences occur, the amino acids coded are noted. Where amino acid replacements occur, the amino acids coded are boxed.

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compared with the spleen and peripheral blood; 2) anti-GA mAbs produced by kidney infiltrating AbPCs appear to result from Ag drive; and 3) anti-GA mAbs derived from these AbPCs are poly-reactive, bind to glomeruli in vivo, and are likely involved in renal injury. Further studies are needed to define the role of AbPCs specific for GAs in the pathogenicity of lupus nephritis.

References


