The Double Edged Sword of the Immune Response

Mutational Analysis of a Murine Anti-Pneumococcal, Anti-DNA Antibody

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Abstract

Anti–double-stranded (ds) DNA antibodies are not only an important diagnostic marker for SLE, but also play an important role in tissue injury. Microbial antigen may be a stimulus for the production of these antibodies. We isolated 99D.7E, an IgG2b monoclonal antibody from a nonautoimmune BALB/c mouse that is cross-reactive with both dsDNA and phosphorylcholine, the dominant hapten on the pneumococcal cell wall. While partially protective against a bacterial challenge, 99D.7E is also pathogenic to the kidney. To identify those molecular motifs that confer on anti-PC antibodies the potential for autoreactivity, we created a panel of 99D.7E mutants with single amino acid substitutions in the heavy chain, and examined the changes in antigen binding and renal deposition. Our results support the hypothesis that charge and affinity for dsDNA are not adequate predictors of the pathogenicity of anti-DNA antibodies. Differential renal damage from anti-dsDNA antibodies may be due to differences in fine specificity, rather than differential affinity for dsDNA. Importantly, high affinity IgG antibodies cross-reactive with bacterial and self antigen exist and can display pathogenic potential, suggesting that defects in peripheral regulation of B cells, activated by foreign antigen but cross-reactive with self antigen, might lead to autoimmune disorders. (_J. Clin. Invest._ 1996. 97:2251–2259.) Key words: anti-DNA antibodies • antibacterial antibodies • cross-reactivity • pathogenicity • systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by anti-double stranded (ds)[1] DNA autoantibodies and immune-mediated damage in many organs

1. _Abbreviations used in this paper:_ ds, double stranded; KLH, keyhole limpet hemocyanin; PC, phosphorylcholine; SCID, severe combined immunodeficient; GBM, glomerular basement membrane.

(1). Immune glomerulonephritis resulting in renal dysfunction is a major cause of morbidity and mortality in this syndrome (2).

It is now clear that anti-dsDNA antibodies directly cause kidney disease (3). Not only have anti-dsDNA antibodies been eluted from the kidneys of patients with SLE and from mice with an SLE-like syndrome, but also non-autoimmune mice harboring transgenes that encode the secreted form of an IgG anti-dsDNA antibody develop glomerulonephritis (4). It remains, however, uncertain how anti-dsDNA antibodies actually cause the renal pathology seen in this disease.

While the titer of anti-dsDNA antibodies in the serum can correlate with the severity of renal disease in SLE, and a rising titer can at times predict a clinical flare (5–7), some patients with SLE have high serum titers of anti-dsDNA antibodies and do not develop kidney disease over many years of follow-up. It is thought that individual characteristics of anti-dsDNA antibodies influence their ability to lead to kidney damage. Among the characteristics of anti-dsDNA antibodies that are thought to promote pathogenicity are IgG isotype, high avidity for dsDNA, cationic charge, expression of particular idiotypic determinants, and perhaps cross-reactivity with glomerular components (3, 8–11). Accurate prediction of the pathogenic capability of anti-dsDNA antibodies could potentially be very important in assisting in difficult therapeutic decisions.

Anti-dsDNA antibodies have the features of antibodies that arise in an antigen-selected immune response (12). However, the initial antigenic stimulus for the production of these autoantibodies remains unclear. Several lines of evidence suggest that microbial antigen may be a stimulus for their production: (_a_) in vitro mutation of an anti-pneumococcal antibody leads to loss of reactivity against pneumococcal antigen, and gain of specificity for dsDNA (13); (_b_) autoimmune NZB mice bred in germ-free conditions and not exposed to environmental microbes have greatly attenuated disease (14); (_c_) protective anti-bacterial antibodies and anti-dsDNA antibodies share idiotypic specificities (15); (_d_) patients with bacterial infection will have transiently elevated titers of anti-dsDNA antibodies that are idiotypically related to the anti-DNA antibodies of patients with SLE (16); and (_e_) cross-reactive antibodies binding both bacterial antigen and dsDNA can be isolated from both humans (17) and mice (18). Some of these cross-reactive antibodies binding both phosphorylcholine (PC), a dominant hapten on the pneumococcal cell wall, and dsDNA, can be protective against a bacterial challenge in vivo, but are also pathogenic and deposit in the kidney (18).

We have isolated a murine cross-reactive IgG anti-PC, anti-dsDNA antibody, and undertaken a structure/function analysis of its binding to both antigens. Our interest in these antibodies arises from our hypothesis that such antibodies may arise routinely by somatic mutation during the course of a normal immune response to bacterial antigen. In an effort to elucidate the characteristics of these protective antibacterial anti-

bodies that render them nephritogenic, we present in this paper an analysis of an in vitro generated B cell genealogy.

Methods

Immunization

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). 6–8-wk-old BALB/c female mice were primed intraperitoneally with 100 ng of purified anti-I-J[d] in complete Freund's adjuvant obtained from Difco (Detroit, MI). The anti-I-J[d] antibody is an IgG2bκ antibody produced by the WF18.2b15 cell line previously described (19). The antibody was purified from ascites fluid using a protein A–Sepharose column. The anti-I-J[d] antibody recognizes a determinant present on a subset of T cells, and immunization with this antibody enables sampling of previously suppressed immune responses (19).

The mice were subsequently immunized with 50 μg of PC linked to keyhole limpet hemocyanin (PC-KLH) intravenously in normal saline. Spleen cells were fused to the nonproducing myeloma cell line NSO by standard hybridoma technology 1 day after PC-KLH immunization, by using a splenocyte to NSO ratio of 5:1 (20). Cells were plated out at a concentration of 2×10^5 NSO cells/ml.

Isolation of the 99D.7E cell line

Culture supernatants from wells containing hybridomas were screened for binding to PC-KLH and dsDNA by ELISA (see below). Cells from wells positive for DNA and PC binding were cloned in soft agar, and clones were screened to confirm antigen binding. The 99D.7E cell line is of the IgG2b isotype, and binds both PC and dsDNA by ELISA. The 99D.7E antibody was tested for its ability to protect SCID mice against a lethal infection with the *S. pneumonia* type 3, WU2 encapsulated strain of pneumococcus as previously described (18).

Sequencing

The nucleotide sequences of the wild type variable region genes and subsequent heavy chain mutants (see below) were determined by the dideoxynucleotide chain termination method using the Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

Isolation of light chain secreting cell line

A cell line secreting only the 99D.7E light chain (99D.7D) was isolated by soft agar cloning of the parental line, followed by overlay with anti–mouse IgG antiserum (21). The light chain only cell line was identified by absence of a precipitate following overlay. Absence of the immunoglobulin heavy chain and continued expression of the light chain was confirmed by standard techniques of ELISA, Western blotting, and RNA dot blot for γ2b and S107 VH gene expression (21).

Oligonucleotide-directed mutagenesis

All PCR reactions were performed in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) using the following program: 1 min at 95°C, 1 min at the appropriate annealing temperature, and 1 min at 72°C for 30 cycles, followed by 10 min at 72 °C. In the primary PCR reactions, 25–50 ng of plasmid DNA and 250 or 500 ng of each of the 5′ and 3′ flanking primers were added. The PCR reaction mix consisted of 1.5 mM MgCl$_2$, 0.l25 mM of each dNTP, 1× buffer for Vent polymerase (10× = 100 mM KCl, 200 mM Tris-HCl pH 8.8, 100 mM (NH$_4$)$_2$SO$_4$, 20 mM MgSO$_4$, 1% Triton X-100) (New England Biolabs, Beverly, MA), and 0.5–1.0 μl of Vent polymerase (New England Biolabs). For the primary PCR reactions, each mutagenic oligomer was used with an appropriate antisense 3′ or sense 5′ V region gene flanking oligomer containing a HindIII or EcoRI restriction site, respectively. The 5′ flanking oligomer is complementary to sequences 300 base pairs 5′

of the V1 genomic DNA. The 3′ flanking oligomer is complementary to sequences 18 base pairs 3′ of JH1. Each pair of PCR products served as templates for the secondary PCR reaction to generate full-length V region genes containing the desired mutation (22). The PCR product of the complete V gene region was 0.9 kb.

Each PCR product was isolated on a 2% agarose gel, and purified with the Spin-bind method (FMC Bioproducts, Rockland, ME). The product was digested with 100 U of HindIII and EcoRI, and then used in a standard ligation reaction with 1 microliter of T4 ligase (New England Biolabs) at 12°C overnight. The wild type and mutant V regions were ligated into the γ2b constant region containing plasmid, p368, a generous gift from Dr. J. Sharon (Boston University, Boston, MA) (23). It contains the murine γ2b constant region gene, Eμ enhancer, the selectable marker *gpt* and ampicillin resistance, and unique HindIII (5′) and EcoRI (3′) sites into which each PCR-generated V gene segment can be inserted.

XL-1 Blue competent *E.coli* bacterial cells (Stratagene, La Jolla, CA) were transformed according to the protocol supplied by the manufacturer, and positive colonies identified by hybridization with a kinased oligonucleotide recognizing sequences in the CDR2 of the heavy chain (5′CCCTTCACAGATGCACTGTAC3′).

DNA transfection

Light chain secreting 99D.7D hybridoma cells were transfected by electroporation with plasmids encoding the wild type and mutant 99D.7E heavy chains. For each transfection reaction, 3×10^6 cells in 0.3 ml PBS and 10 μg of linearized plasmid DNA were used. Circular plasmid DNA was linearized with Bgl II or EcoRI. Electroporation conditions were R = infinity, 600 mV at 25 microfarad. After electroporation, cells were resuspended in 10 ml of selective (HX) medium (1% L-glutamine, 1% penicillin-streptomycin, 1% nonessential amino acids, 0.003% amphotericin B, 10 mM Hepes, pH 7.4, 0.15 mg/ml hypoxanthine, and 0.25 mg/ml of xanthine (Sigma Chemical Co., St. Louis, MO), brought up to volume with Iscove's modified Dulbecco's Medium (GIBCO BRL, Life Technologies, Gaithersburg, MD), supplemented with 20% fetal calf serum (Hyclone Labs, Logan, UT) and 10% supernatant from Con A - stimulated spleen cells, and plated into 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ). After incubation at 37°C for 24–48 h, 4 μg/ml of mycophenolic acid (Sigma Chemical Co.) was added to the culture medium. Surviving clones were screened by ELISA for secretion of the heavy chain. ELISA assays confirmed that each transfectant co-expressed both heavy and light chain determinants in a single antibody molecule (21).

Quantitation of antibodies

96-well microtiter ELISA plates (Becton Dickinson) were coated with a 1:1000 dilution of goat anti–mouse IgG2b (Fisher Scientific Co., Pittsburgh, PA) in PBS at 4°C overnight. Wells were blocked with 3% fetal calf serum in PBS, washed extensively with PBS/0.05% Tween 20, and incubated for 1 h at 37°C with serial dilutions of culture supernatants, serum from tumor-bearing mice, or two fold dilutions of a commercially purified IgG2b standard (MOPC 141; Sigma Chemical Co.) at 500 ng/ml initial dilution. After washing with PBS/0.05% Tween 20, wells were incubated with a 1:1000 dilution of alkaline-phosphatase linked goat anti–mouse IgG2b (Fisher Scientific) for 1 h. After a final wash, the reactions were developed with alkaline phosphatase substrate (*p*-nitrophenyl phosphate disodium tablets) (Sigma Chemical Co.). The reactions were monitored at 405 nm in a Titertek ELISA reader.

ELISA assays for antigenic specificity

dsDNA. Calf-thymus DNA was dissolved in PBS at 4°C and filtered though a 0.45 μm nitrocellulose Millex syringe filter (Millipore Corp., Bedford, MA) to obtain dsDNA. One hundred microliter of dsDNA at 50 μg/ml was added to Immulon II round-bottom polyvinyl 96-well plates (Dynatech Laboratories, Chantilly, VA), and dried overnight at 37°C. Normalized supernatants were incubated for 90 min at 37°C,

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         1                                                                                                              90
V1  GAG GTG AAG CTG GTG GAA TCT GGA GGA GGC TTG GTA CAG CCT GGG GGT TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT
     E   V   K   L   V   E   S   G   G   G   L   V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   F   T   F   S
     1                           10                              20                                      30
99D.7e --- C-- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- ---
        -   L   N   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   P   -   -   -   -   -

         91                                                                                                             180
V1  GAT TTC TAC ATG GAG TGG GTC CGC CAG CCT CCA GGG AAG AGA CTG GAG TGG ATT GCT GCA AGT AGA AAC AAA GCT AAT GAT TAT ACA ACA
     D   F   Y   M   E   W   V   R   Q   P   P   G   K   R   L   E   W   I   A   A   S   R   N   K   A   N   D   Y   T   T
                                    40                                      50          a   b   c
99D.7e --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- GC- --- --- --- --- --- --- ---
        -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   A   -   -   -   -   -   -   -

V1  GAG TAC AGT GCA TCT GTG AAG GGT CGG TTC ATC GTC TCC AGA GAC ACT TCC CAA AGC ATC CTC TAC CTT CAG ATG AAT GCC CTG AGA GCT
                                                                                                                        270
     E   Y   S   A   S   V   K   G   R   F   I   V   S   R   D   T   S   Q   S   I   L   Y   L   Q   M   N   A   L   R   A
             60                              70                                  80              a   b   c
99D.7e --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- C--
        -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   P

         271                               303                 JH1
V1  GAG GAC ACT GGC ATT TAT TAC TGT GCA AGA GAT  -- --- --- --- --- --- TAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC
     E   D   T   G   I   Y   Y   C   A   R   D    -   -   -   -   -   -   -   Y   W   Y   F   D   V   W   G   A   G   T   T   V
                         90              95                                                          103
99D.7e --- --- --- --- --- --- --- --- --- --- --..  GA GGG GGC TTA TAT GTT ... --- --- --- --- --- --- --- --- --- --- --- ---
        -   -   -   -   -   -   -   -   -   -   .     G   G   G   L   Y   V   .   -   -   -   -   -   -   -   -   -   -   -

V1  ACC GTC TCC TCA
     T   V   S   S
             113
99D.7e --- --- --- ---
        -   -   -   -
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Figure 1. Nucleotide and deduced amino acid sequence of the heavy chain of the 99D.7E antibody encoded by the V1 of the S107 VH family, compared with the germline V1 gene.

followed by secondary antibody goat anti-mouse kappa light chain at a 1:1000 dilution (Southern Biotechnology Associates, Birmingham, AL). Assay development then proceeded as described above for antibody quantitations.

PC. 96-well microtiter ELISA plates (Becton Dickinson) were coated with 100 μl of PC-KLH at 20 μg/ml at 4°C overnight. The ELISA for PC binding was then performed as described above for antibody quantitations.

Renal pathogenicity studies

SCID mice were obtained from the breeding colony maintained at the Albert Einstein College of Medicine. Six to 8-wk-old female mice

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         1                                                                                                              90
V9  GAC ATC CAG ATG ACA CAG TCT CCA TCC TCA CTG TCT GCA TCT CTG GGA GGC AAA GTC ACC ATC ACT TGC AAG GCA AGC CAA GAC ATT AAC
     D   I   Q   M   T   Q   S   P   S   S   L   S   A   S   L   G   G   K   V   T   I   T   C   K   A   S   Q   D   I   N
     1                           10                              20                                      30
99D.7e --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

         91                                                                                                             180
V9  AAG TAT ATA GCT TGG TAC CAA CAC AAG CCT GGA AAA GGT CCT AGG CTG CTC ATA CAT TAC ACA TCT ACA TTA CAG CCA GGC ATC CCA TCA
     K   Y   I   A   W   Y   Q   H   K   P   G   K   G   P   R   L   L   I   H   Y   T   S   T   L   Q   P   G   I   P   S
                                    40                                      50                                      60
99D.7e --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

         181                                                                                                            270
V9  AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC TTC AGC ATC AGC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT TGT CTA CAG
     R   F   S   G   S   G   S   G   R   D   Y   S   F   S   I   S   N   L   E   P   E   D   I   A   T   Y   Y   C   L   Q
                                    70                                      80                                      90
99d.7e --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --C --- --- --- --- --- --- ---
        -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -

         271                         JK1
V9  TAT GAT AAT CTT CTG  TGG ACG     TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA
     Y   D   N   L   L   W   T       F   G   G   G   T   K   L   E   I   K
                                                                      107
99D.7e --- --- --- --- ---  --- ---     --- --- --- --- --- --- --- --- --- ---
        -   -   -   -   -   -   -       -   -   -   -   -   -   -   -   -   -
```

Figure 2. Nucleotide and deduced amino acid sequence of the 99D.7E light chain variable region compared with the germline Vκ9 and Jκ1 sequence.

were primed with 0.5 ml pristane (tetra-methyl-pentadecane, Sigma) intraperitoneally. One week later, the pristane injection was repeated. The following day, 10^7 cells in sterile saline were injected intraperitoneally. The mice were bled and then sacrificed 10–14 d after ascites or tumors developed, and the kidneys were removed for fixation. Proteinuria was estimated by examination of fresh urine using Chemstrip (Boehringer Mannheim, Indianapolis, IN), on a scale of 0 to 3+, where 0 is negative or trace, 1+: 30 mg/dl; 2+: 100 mg/dl; and 3+: 500 mg/dl protein. Serum IgG2b levels were quantitated as described above.

One kidney from each animal was fixed in 10% formalin and embedded in paraffin to analyze for immunoglobulin deposition. 4-μm-thick sections were obtained by microtome, deparaffinized, rehydrated, blocked with 2% BSA in PBS in moist chambers, and stained for 1 hour with biotinylated goat anti–mouse IgG at a 1:800 dilution at room temperature (Vector Laboratories, Inc., Burlingame, CA). The sections were washed, incubated for 45 min with alkaline phosphatase labeled ABC reagent (Vecta stain ABC kit) and developed with substrate for streptavidin-alkaline phosphatase (BCIP, 5 Bromo-4 chloro-3 indoyl phosphate-*p*-toluidine salt and NBT, Nitroblue Tetrazolium Chloride substrate, GIBCO BRL). The color development was stopped by the addition of distilled water. The sections were mounted on coverslips with cytosol mounting medium (Aqua polymount, Polysciences Inc., Warrington, PA), sealed and viewed with a Zeiss microscope.

Kidney sections were also stained by standard techniques with hematoxylin and eosin, for histological examination and correlation with the immunochemistry findings. Findings were reported according to the percentage of abnormal glomeruli, glomerular cellularity and infiltration by inflammatory cells, and presence of tubular damage as signified by vacuolar changes.

Both immunochemistry and histology slides were read blindly, without knowledge of the cell line injected or serum IgG2b level.

Results

Characterization of 99D.7E. The heavy chain of the 99D.7E antibody is encoded by the S107 V1, an unknown D, and the JH1 gene segments; the light chain by Vκ9 and Jκ1. The VH gene of 99D.7E is 98% homologous to the V1 germline gene. There are six nucleotide differences, three in the FR1, two in the CDR2, and one in the FR3. These differences lead to the replacement of five amino acids: valine to leucine at position 2, lysine to asparagine at position 3, and threonine to proline at position 24 in FR1; lysine to alanine at position 52b in CDR2; and alanine to proline at position 84 in FR3 (Fig. 1). Two nucleotides that encode for the amino acid at position 95 (accord-

Table I. Assays for Antigenic Specificity

	ELISAs for dsDNA and PC binding	
	dsDNA	PC-KLH
	OD	*OD*
Positive control	0.64±0.05	1.33±0.17
Negative control	0.06±0.002	0.07±0.01
99D.7E	1.58±0.06	1.50±0.06

The controls for the assays were: dsDNA, positive control, R4A, an IgG2b anti-dsDNA antibody; and negative control, 3U12.7, a non-DNA binding antibody of the same isotype; for PC, positive control, PCγ, a canonical anti-PC antibody (IgG2a) (a gift from M. Scharff, Bronx, NY); and negative control, an irrelevant IgG antibody. Cell lines were grown in serum-free media, and antibodies were normalized to 4 μg/mg for the PC-KLH ELISA, and 5 μg/ml for the dsDNA ELISA. OD signifies optical density at 405 nm. The reported results are representative of several assays.

ing to Kabat) in the VH region are missing in the sequence, so the deduced amino acid sequence of 99D.7E does not contain an amino acid at this position. The 99D.7E light chain is identical to the germline Vκ9, without any mutations (Fig. 2).

99D.7E binds both dsDNA and PC by ELISA assay (Table I). Using the method of Nieto et al (24), the calculated affinity of 99D.7E for PC is one to two logs lower than the canonical T15+ anti-PC (PCγ) antibody (data not shown). The 99D.7E antibody also binds dsDNA by Millipore filter assay (data not shown). Furthermore, 99D.7E demonstrated partial protection against an in vivo bacterial challenge with virulent pneumococci. The degree of protection afforded by the 99D.7E antibody was less than that afforded by the canonical PCγ anti-PC antibody, but greater than saline alone (Fig. 3). The survival of mice pretreated with the 99D.7E antibody was significantly improved compared to mice with no antibody pretreatment as calculated by the log-rank test ($P = 0.0018$).

Generation and characterization of 99D.7E mutants. The 99D.7E heavy chain was subjected to site-directed mutagenesis to study the effects of amino acid replacements on antibody affinity to PC and dsDNA, and on specificity and pathogenicity. Mutations were made based on sequence comparisons of
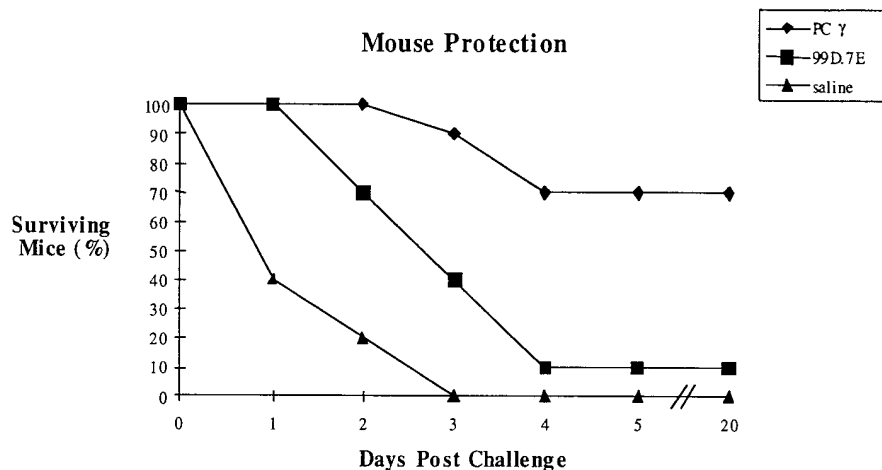

Figure 3. Mouse protection assay. 6–8-wk-old SCID mice were injected with 500 WU2 pneumococci intraperitoneally. Animals were monitored twice daily for survival over 20 d. No further mortality after day three was seen in the mice injected with PCγ and 99D.7E antibodies. Data in the graph are representative of two experiments. Survival of mice treated with 99D.7E was significantly better than mice with the saline pretreatment ($P = 0.0018$).

S107 VH encoded anti-PC antibodies, and anti-dsDNA antibodies. Several mutants (44, 52, 66) were designed to test the importance of arginine in dsDNA specificity. In other mutants (31, 52a, 85), substitutions were intended to change the charge of the V region. Mutations were generated both in FRs (2, 33, 66, 85) and in CDRs (31, 34, 52, 52a) to assay the relative contribution of these regions to dsDNA binding.

Two mutations resulted in no change in dsDNA binding, a methionine to isoleucine change in residue 34 in CDR1, and a glutamic acid to valine substitution in residue 85 in FR3. Three substitutions resulted in only a mild decrease in dsDNA binding, a change from leucine (residue 2) to valine in FR1, aspartic acid (residue 31) to valine in CDR1, and arginine to glycine in residue 52 in CDR2. Greater decreases in dsDNA binding occurred with a mutation at residue 44 (FR2), an arginine to valine substitution, and at residue 52a (CDR2), an asparagine to serine substitution. A substitution of a leucine for an arginine at residue 66 in FR3 resulted in a marked increase in dsDNA binding (Table II).

Most mutations in the 99D.7E heavy chain leading to an increase (antibody 99D.66) or decrease (antibody 99D.2, 44, 52, 52a) in dsDNA binding resulted in a similar change in PC reactivity, in both magnitude and direction (Table II). Similarly, antibody 99D.85, with a glutamic acid to valine substitution in residue 85 in FR3, showed no change in PC or dsDNA binding. The 99D.31 antibody (aspartic acid to valine substitution in residue 31 in CDR1) displayed a mild decrease in dsDNA binding but a marked decrease in PC reactivity, while the 99D.34 antibody (methionine to isoleucine substitution in residue 34 in CDR1) showed no change in binding to dsDNA, but a marked increase in binding to PC.

Renal deposition and pathogenicity. The wild-type 99D.7E antibody and four mutated antibodies 99D.31, 44, 52a, and 66 were assayed for renal pathogenicity after intraperitoneal hybridoma instillation. An alternative method to investigate the pathogenicity of anti-DNA antibodies is intraperitoneal or intravenous injection of purified antibodies. However, the disadvantage of this method is that antibody purification involves acid or alkali elution from an affinity column, which results in some denaturation of the antibody. The exact degree of denaturation cannot be determined, and may very well vary among different antibodies. We consequently chose a method in which the antibody is produced in vivo so no denaturation occurs, and compared mice with equivalent titers of serum antibody levels. Most of the mice harboring antibody-producing tumors showed moderate amounts of proteinuria, averaging +2 on the semiquantitative assay for protein. For the purpose of comparing the severity and localization of immunoglobulin deposits, the mice were divided into two groups: group 1 (99D.7E, 99D.44) with high serum IgG2b immunoglobulin levels of 1.5–2 mg/ml (Fig. 4 *A*) and group 2 (99D.31, 52a, 66) with serum IgG2b levels of 20–30 μg/ml (Fig. 4 *B*). In group 1, mice injected with the 99D.7E cell line showed very intense glomerular binding (Fig. 4 *A 2*), without any significant tubular involvement. While some glomerular immunoglobulin deposits were present in mice injected with 99D.44, tubular deposition was more prominent (Fig. 4 *A 3*). In group 2, mice injected with 99D.52a and 99D.66 had dense glomerular and tubular deposits (Fig. 4 *B, 1* and *2*), while at similar serum IgG2b concentrations no renal deposition was seen in mice injected with 99D.31 (Fig. 4 *B 3*). While a direct comparison cannot be done because of the disparity in immunoglobulin levels, it is interesting to note that mice injected with 99D.52a and 99D.66 had significant tubular deposits at relatively low immunoglobulin levels, while mice injected with the wild type antibody had little tubular immunoglobulin deposition even with a three-log-old greater level of serum IgG2b.

Pristane itself has an inflammatory effect. It has been recently reported that some non-autoimmune BALB/c mice receiving an intraperitoneal injection of pristane can develop autoantibodies and glomerulonephritis (25). However, in this study we have generated ascites in SCID mice, which will not develop endogenous autoantibodies. Moreover, both our negative controls (the nonpathogenic antibody 99D.31 and no antibody injected) received pristane, and no immunoglobulin deposition was observed.

Findings on light microscopy showed good correlation with immunochemical staining for immune deposits. Findings on light microscopy for the 99D.7E cell line showed mild to moderate hypercellularity, with 25–50% of the glomeruli involved, and minimal tubular vacuolar changes. Mice injected with the 99D.52a cell line showed involvement of 50% of glomeruli, with moderate hypercellularity and vacuolar change, while mice injected with 99D.44 showed pathology predominantly in

Table II. Characterization of 99D Mutants

Cell line	Mutation; residue	dsDNA	PC	Pathogenicity
		OD	*OD*	
wt (99D.7E)		1.27±0.01	1.95±0.00	+, glomerular
99D.2	Leucine to valine, residue 2 (FR1)	0.94±0.1	1.60±0.09	ND
99D.31	Aspartic acid to valine, residue 31 (CDR1)	0.84±0.06	1.07±0.03	—
99D.34	Methionine to isoleucine, residue 34 (CDR1)	1.20±0.05	2.44±0.01	ND
99D.44	Arginine to valine, residue 44 (FR2)	0.41±0.01	0.57±0.04	+, tubular
99D.52	Arginine to glycine, residue 52 (CDR2)	0.86±0.08	1.63±0.15	ND
99D.52a	Asparagine to serine, residue 52a (CDR2)	0.61±0.02	1.15±0.08	+, glomerular, tubular
99D.66	Arginine to leucine, residue 66 (FR3)	2.19±0.1	2.55±0.7	+, glomerular, tubular
99D.85	Glutamic acid to valine, residue 85 (FR3)	1.32±0.09	2.12±0.1	ND

Binding to dsDNA, PC, and assay for pathogenicity for the 99D.7E and in vitro generated mutants. OD signifies optical density at 405nm, ND signifies not done.
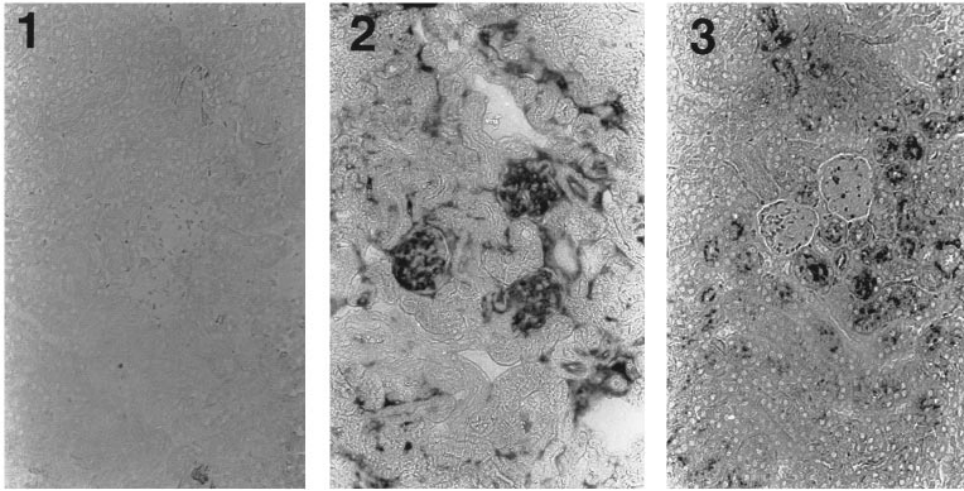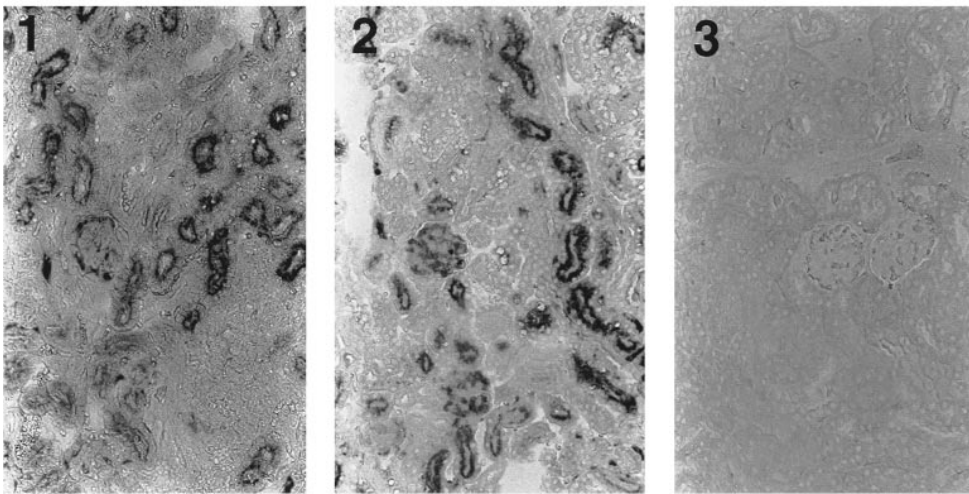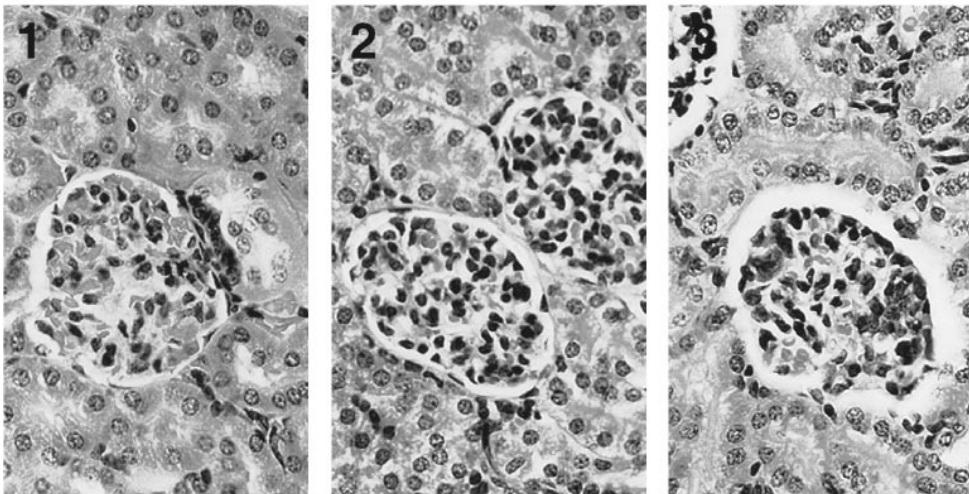
Figure 4. (*A* and *B*) Immunoglobulin kidney deposition. SCID mice were pristane-primed twice, injected with 10^7 hybridoma cells, and sacrificed when ascites or tumors developed. Kidneys were fixated, sectioned, and stained with biotinylated anti-mouse immunoglobulin, followed by streptavidin-alkaline phosphatase and alkaline phosphatase substrate. (*A, 2–3*) serum IgG2b levels of 1.5–2 mg/ml; (*B, 1–3*) serum IgG2b levels of 20-30 μg/ml. (*A 1*) Negative control, pristane-primed mice (no cell line injected). *A 2,* 99D.7E; *A 3,* 99D.44; *B 1,* 99D52a; *B 2,* 99D.66; *B 3,* 99D.31. Glomerular immune deposits are seen for 99D.7E while tubular deposits are present in mice injected with 99D.44. Both tubular and glomerular deposits are present in mice given 99D.52a and 99D.66. No IgG deposition is observed for mice receiving only pristane injections, or those receiving the 99D.31 cell line. (*C*) Hematoxylin and eosin staining (light microscopy). (*C 1*) Normal glomerulus and tubules from a pristane primed mouse. (*C 2*) 99D.7E, showing moderate hypercellularity and minimal tubular vacuolar change. (*C 3*) 99D.66, again showing moderate hypercellularity, and more pronounced tubular vacuolar damage.

the tubules. The most severe glomerular damage was found with the 99D.66 antibody, with 75% of glomeruli affected, moderate to severe hypercellularity, with mild tubular changes. It is important to note that chronic nephrotoxicity is not likely to develop in the relatively short time from injection of the hybridoma cells until the kidneys were examined, and therefore this was not quantitatively assessed.

Discussion

Microbial antigen is postulated to be a trigger for the production of anti-dsDNA antibodies. One hypothesis which has been put forward is that autoreactivity may arise as the result of somatic hypermutation of protective, anti-bacterial antibodies. In one in vitro study, a single amino acid replacement in CDR1 of the heavy chain of an anti-pneumococcal antibody led to loss of reactivity with PC, with gain of specificity for ds-DNA (13). We have previously isolated a number of cross-reactive IgM anti-PC, anti-dsDNA antibodies from non-autoimmune mice (18, 26), and have suggested that cross-reactive antibodies may be routinely generated during the course of a normal immune response to a foreign antigen. These cross-reactive antibodies do not usually contribute to the expressed antibody repertoire, because B cells displaying autospecificities are destined for anergy or apoptosis and are not sampled using conventional hybridoma technology (27). Since the cross-reactive anti-PC, anti-dsDNA antibodies we have reported previously were of the IgM isotype, the dual specificity might conceivably be a consequence of higher avidity of the IgM molecule, which occurs when the antibody is present in a pentameric form. Here, for the first time, we report a dual specific IgG antibody with nephritogenic potential. Molecular analysis of this antibody is then of special importance.

The nucleotide sequences contributing to DNA binding have been a subject of intense study in recent years (12, 28–30). The basic amino acids arginine (R) and lysine (K) are capable of interacting with DNA or with its deoxyribose phosphate backbone. This provides an explanation for why during the process of affinity maturation of anti-dsDNA antibodies many somatic mutations lead to the acquisition of one of these amino acids. Computer models of anti-DNA antibodies have suggested that arginine, glutamine or tyrosine project from the CDRs of the immunoglobulin molecule into the antigen binding groove, where they contribute to high avidity binding (31). In particular, arginine residues in heavy chain CDR3 have been implicated in dsDNA binding, although about one-third of all anti-dsDNA antibodies have no arginine at all in CDR3 (21). Negatively charged amino acids (aspartic acid [D], and glutamate [E]) are also thought to be more prevalent in CDRs of pathogenic anti-dsDNA antibodies (8).

Several of our mutated antibodies seemed to support these general conclusions. Loss of an aspartic acid in CDR1 (99D.31), arginine in CDR2 (99D.52) and asparagine in CDR2 (99D.52a) each caused a decrease in binding to dsDNA. Loss of an arginine in FR3 in antibody 99D.66, which might be predicted to cause no change or a decrease in DNA binding as it is unlikely to contribute at this position to the antibody binding site, unexpectedly resulted in a marked increase in affinity for dsDNA. Interestingly, a loss of an arginine in the same position in a S107 V11 encoded anti-dsDNA antibody also resulted in a marked increase in dsDNA binding (21).

A more surprising and important finding in our study is the clear dissociation between in vitro dsDNA binding as assayed by ELISA, and the observed pathogenicity of the antibody in vivo. Antibodies 99D.44 and 52a, with significant decreases in binding to dsDNA, nevertheless were pathogenic and caused immunoglobulin deposition in the kidneys. Interestingly, the mutations not only lead to a change in affinity for dsDNA, but also in the anatomic localization of the renal deposition. Both these antibodies deposited in the tubules, in contrast to the purely glomerular deposition of the parent 99D.7E.

Of note is the intense tubular deposition seen in the mutant antibodies 99D.44, 52a, and 66. While predominantly tubular deposition is not common in human lupus nephritis (32), immune deposits may be present in tubular basement membranes and interstitium in 50% of kidney biopsies from patients with SLE (33, 34). In a study by Park et al. (35), the prevalence of tubular immune deposits correlated with activity and severity of glomerular lesions, as well as with the degree of renal insufficiency. Importantly, tubular immune deposits were also found to be prognostically indicative of progressive renal deterioration.

The precise anatomical location of the tubular deposition observed with these antibodies has not been determined, and cannot be determined with the degree of resolution available in immunofluorescence studies. One possibility that should be considered is that the tubular deposits are present within the brush border, rather than along the tubular basement membrane. Such a pattern can occur in myeloma, due to the uptake of immunoglobulins by the tubular cells. In multiple myeloma, however, the serum levels of the monoclonal paraproteins are on the order of tens of grams per liter, while we examined mice with immunoglobulin levels at least a log lower. In addition, mice injected with the wild type 99D.7E antibody had just as high serum antibody levels as the mice injected with the tubular-depositing antibody 99D.44, yet no tubular deposition was present. This suggests that tubular immunoglobulin deposition was related to the fine specificity of the antibody, rather than a nonspecific consequence of hypergammaglobulinemia.

The pathogenesis of nephritis in SLE is a subject of much debate, and it is unclear how anti-dsDNA antibodies actually cause renal disease. Early work in the field attributed kidney damage to the passive entrapment of DNA-anti-DNA immune complexes in the kidney (36). However, it has not been possible to demonstrate routinely the presence of DNA, either free in the circulation or bound in immune complexes, in patients with SLE (37). DNA-anti-DNA complexes prepared ex vivo and injected into animals showed no affinity for glomerular basement membrane (GBM), and were rapidly cleared from the circulation by the reticuloendothelial system (38). In addition, it seems unlikely that the negatively charged DNA in the immune complex could bind easily to the negatively charged GBM. These results and others led to focusing on possible cross-reactions of anti-DNA antibodies with glomerular components. Many potentially relevant anti–DNA-GBM interactions have been described, including binding of anti-DNA antibodies to fibronectin, collagen, and laminin (3). Recently, a monoclonal anti-DNA antibody from a lupus prone MRL-1pr mouse was found to bind to a protein with homology to the SPARC/osteonectin family of extracellular matrix proteins (39). Several investigators have found that a subset of anti-DNA antibodies cross-reacts with heparan sulfate, an important component of the GBM (40–42). Furthermore, interfering

with the putative cross-reactivity of anti-DNA antibodies with heparan sulfate by injection of heparin was shown to ameliorate kidney disease in lupus prone mice (43). Alternatively, it has been suggested that anti-DNA antibodies bind the basement membrane indirectly, by recognizing histone-DNA complexes that adhere to GBM collagen (44).

Our results support the hypothesis that cross-reactivity of anti-DNA antibodies with kidney components is a central mechanism in the pathogenesis of lupus nephritis, and confirm previous findings from this laboratory. Katz et al. studying a different antibody found, as did we, that a single amino acid change in the V region of an anti-DNA molecule is sufficient to alter the in vivo localization of deposition of the pathogenic antibody (21). It seems unlikely that DNA is differentially present in the glomeruli or tubules, or that DNA/anti-DNA complexes are harbored differently at these sites. Therefore, cross-reactivity with a glomerular or tubular antigen may be a major determinant of pathogenicity.

It is interesting to compare the results of our PC-binding assays of the mutated antibodies with other published anti-PC sequences and with crystallographic data. In the anti-PC antibody McPC603, PC was found to bind in a pocket bounded by Tyr 94 (light chain CDR3), Tyr 33 (heavy chain CDR1), Arg 52 (heavy chain CDR2) and Trp 100 (heavy chain CDR3) (45). In the heavy chain of 99D.7E, the tyrosine, tryptophan and arginine are conserved. The arginine to glycine change in residue 52 would be predicted to decrease PC binding, as it actually does. Several murine anti-PC antibodies of the S107 family have a DFYMEW sequence in their CDR1 (46); replacing the aspartic acid in residue 31 decreases PC binding, suggesting that this particular CDR1 sequence is indeed important for PC binding. Replacement of the methionine with isoleucine in residue 34 increases PC binding; HPCG 10 has an identical CDR1 sequence to the mutated antibody 99D.34 and also binds PC (47), but the affinity for PC relative to 99D.7E is unknown. Residue 85 has no known function in binding to PC; so replacement of a glutamic acid with valine, as expected, does not affect PC binding. Framework mutations at position 44 and 66 are additional examples of the contribution of framework residues to antigen binding. Replacement in framework regions can enhance (antibody 99D.66) or diminish (antibody 99D.44) both PC and dsDNA binding, either by direct interaction with the antigen, or through distal conformational effects. The leucine to valine change in residue 2 is a mutation back to the germline V1 heavy chain. While changes back to germline might be theorized to decrease DNA binding and increase PC binding, direct comparison with the T15+ antibody is not possible because of the different VDJ junctions and different light chain usage.

Our results have implications toward the understanding of immune regulation in non-autoimmune individuals. Somatic mutation can presumably lead to the generation of antibodies with a newly acquired autospecificity but with a lower affinity to foreign antigen than the canonical response, such as the U4, the S107 variant reported several years ago (13). U4 analogs that arise in vivo will not be amplified due to the lower affinity for the triggering foreign antigen. However, high affinity, class switched IgG cross-reactive antibodies can also be generated by only a small number of amino acid substitutions, as shown by the 99D.7E cell line and its in vitro genealogy. Some of these mutations increase affinity for the triggering foreign antigen (antibodies 99D.34 and 66), yet in 99D.66 this also clearly

led to a pathogenic antibody. Moreover, pathogenicity in vivo does not always correlate with the avidity for the identified self-antigen; antibodies can be pathogenic despite low avidity for dsDNA (antibody 99D.44).

A study just published by Casson and Manser (48) has confirmed our results in a different antibody system. By random mutagenesis of two positions in VH CDR2 of an anti-*p*-azophenylarsonate antibody, it was found that while a significant number of mutated antibodies had higher affinity for azophenylarsonate than the wild type V region, 9 of 11 of the high affinity mutant antibodies also bound strongly to denatured DNA. Taken together with our data, these results seem to indicate that in the context of B cell maturation and selection in germinal centers, both positive and negative selection forces must be operative. Positive selection alone would increase the pool of B cells with higher affinity to the selecting antigen, but may also result in expansion of the cross-reactive B cells with higher affinity for foreign antigen which have also acquired a new anti-self specificity. It seems likely that only through application of both positive and negative selection forces during B cell development can the immune system reliably mature the anti-foreign repertoire, while minimizing the destructive potential of acquired autoreactivity.

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