Mutational Analysis of an Autoantibody: Differential Binding and Pathogenicity

By Jessica B. Katz, Wacharee Limpanasithikul, and Betty Diamond

From the Departments of Microbiology and Immunology, and Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Summary

We have used site-directed mutagenesis to change amino acid residues in the heavy chain of the pathogenic R4A anti–double-stranded DNA (dsDNA) antibody and have looked for resultant alterations in DNA binding and in pathogenicity. The data demonstrate that single amino acid substitutions in both complementarity determining and framework regions alter antigen binding. Changes in only a few amino acids entirely ablate DNA specificity or cause a 10-fold increase in relative binding. In vivo studies in mice of the pathogenicity of the mutated antibodies show that a single amino acid substitution leading to a loss of dsDNA binding leads also to a loss of glomerular sequestration. Amino acid substitutions that increase relative affinity for dsDNA cause a change in localization of immunoglobulin deposition from glomeruli to renal tubules. These studies demonstrate that small numbers of amino acid substitutions can dramatically alter antigen binding and pathogenicity, and that the pathogenicity of anti-DNA antibodies does not strictly correlate with affinity for DNA.

Anti–double-stranded DNA (dsDNA)[1] antibodies are present in the serum of patients with SLE. The fluctuation in titer of these antibodies in association with disease activity (1) and their presence in the kidneys of lupus patients (2) have led investigators to believe they are involved in the pathogenesis of the disease, specifically in the development of lupus glomerulonephritis. Over the past several years, a number of studies of the molecular genetics and protein structure of anti-dsDNA antibodies have been undertaken as a way of trying to understand: (*a*) whether the anti-DNA response in SLE is elicited by antigen, and, if so, whether that antigen is DNA, another self antigen, or a foreign antigen; (*b*) what particular features of these antibodies account for DNA binding; and (*c*) what are the determinants of pathogenicity of anti-DNA antibodies (3–6).

Mutational analyses of anti-DNA antibodies have been performed to try to elucidate whether DNA binding specificity represents part of an antigen-selected response. Clustering of replacement (R) base changes that lead to an amino acid change in CDRs is one measure commonly used to suggest antigen selection (7). This is clearly based on the primacy of CDRs in determining antigen specificity and affinity. Second, the ratio of R mutations to silent (S) base changes that do not lead to a change in amino acid, has been calculated. R/S ratios greater than random are thought to be evidence for antigen selection. B cell genealogies have been especially useful in these analyses as they identify those mutated progeny of a single B cell that are amplified in vivo. Whereas several studies have been interpreted as showing the anti-DNA response is antigen driven (3, 4, 6, 8, 9), other studies have shown that many anti-DNA antibodies do not meet criteria for antigen selection and have raised the question whether other forces are operative in the anti-DNA response (10).

Sequence analysis of anti-dsDNA antibodies has led to the speculation that certain amino acids are present in the CDRs of anti-DNA antibodies at a higher frequency than in antibodies with other antigenic specificities. These amino acids include arginine, lysine, tyrosine, glutamine, and asparagine. In particular, arginine residues in the H chain have been implicated in dsDNA binding (4) as arginine has the potential to interact with the phosphodiester backbone of dsDNA or with cytosine-guanine base pairs (11). Whereas the presence of arginine residues in the CDR3 is striking, it is important to recognize that approximately one third of anti-dsDNA antibodies have no arginine in CDR3. Furthermore, the recent crystallographic analysis of an antitrithymidilate antibody (12) suggests that many amino acid residues participate in binding to DNA.

Finally, the determinants of pathogenicity of anti-DNA antibodies have been examined. It is clear that cationic anti-DNA antibodies are, in general, more likely to deposit in glomeruli than neutral or anionic antibodies (13), yet not all cationic anti-DNA antibodies are pathogenic. Some studies have suggested that pathogenic anti-DNA antibodies cross-react with tubular antigens since lupus nephritis may include tubular as well as glomerular disease (14, 15). It has been suggested that tubular deposition of Ig occurs when immune complexes are large, whereas smaller complexes cause glo-

[1] *Abbreviations used in this paper:* dsDNA, double-stranded DNA; FR, framework region; R/S ratio, ratio of replacement mutations to silent base changes; ss, single-stranded DNA.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/09/0925/08 $2.00
Volume 180 September 1994 925–932

merular deposition only. Clearly, the determinants of renal sequestration and pathogenicity are not yet defined at a molecular level.

We present in this paper an analysis of in vitro–generated mutants of a murine anti-dsDNA antibody. We selected to delete or insert arginines to test the importance of this amino acid in DNA binding. Mutations were made in both CDRs and framework regions (FRs). We wished also to ask whether a change in charge of the V region was sufficient to alter DNA binding or whether only changes in charge within the antigen binding site would alter antigen binding. In two mutants we removed a negatively charged residue to test whether these also contribute to dsDNA binding as has been suggested by Ohnishi et al. (16). The relative binding to DNA of the mutant antibodies was measured. In addition, the renal sequestration of some of these antibodies was determined in SCID mice.

Materials and Methods

Isolation of a L Chain–secreting R4A Cell Line. The R4A cell line was derived from a BALB/c mouse immunized with phosphorylcholine (PC) conjugated to human gamma globulin (17). The secreted antibody binds dsDNA on a filter assay (Millipore Corp., Bedford, MA) and on an ELISA, and is encoded by the unmutated germline S107 V11 H chain gene and an unmutated germline $V_\kappa 1$ L chain gene.

A cell line secreting only the R4A L chain was isolated by soft agarose cloning and overlayed with rabbit anti-mouse IgG antiserum kindly provided by Dr. Matthew Scharff (Albert Einstein College of Medicine) (18). Absence of Ig H chain expression in selected clones was confirmed by RNA dot blot using a probe that hybridizes to the H chain V region (19). Continued expression of the R4A L chain was ascertained by an ELISA for κ L chain in culture supernatant and RNA dot blot of cells using a $V_\kappa 1$ probe that hybridizes to the R4A L chain.

Oligonucleotide-directed Mutagenesis. A plasmid containing an EcoRI-EcoRI 3.5-kb rearranged V region gene of the R4A H chain (pBluescript SK; Stratagene Cloning Systems, La Jolla, CA) was used as the template for the mutagenic PCR reactions (20). All PCR reactions were performed in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using the following program: 1.5 min at 96°C and 3.5 min at 72°C for 30 cycles. The primary reactions, 25–30 ng of plasmid DNA and 250 ng of each primer were used. In the secondary PCR reaction, 1% of the two primary reactions and 250 ng of each of the 5′ and 3′ flanking primers were added. The PCR reaction mix consisted of 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 1× Taq buffer (10× = 100 mM Tris-HCl, pH 8.3, 500 mM KCl, and 0.1% gelatin), and 1 μl Taq polymerase (Perkin Elmer Cetus). 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 site, respectively, plus a GGG clamp. The 5′ flanking oligomer is complementary to sequences 500 bp 5′ of the V11 genomic DNA. The 3′ oligomer is complementary to sequences 30 bp 3′ of J_H4. Each pair of PCR products served as templates for the secondary reaction to generate full-length V region genes containing the desired mutations. One PCR reaction generated the wild-type R4A H chain sequence as a control for all subsequent analyses.

Each PCR product was isolated from a 0.8% agarose gel and purified through an Elutip-D minicolumn (Schleicher & Schuell, Inc., Keene, NH). After digestion with 50–100 U of HindIII and EcoRI, the PCR product was used in a standard ligation reaction.

Plasmid Construction. The γ2b C region and gpt-containing plasmid, p368, was generously provided by Dr. Jacqueline Sharon (Boston University, Boston, MA) (21). It contains the Cγ2b gene, Ig H chain enhancer, and the selectable marker *gpt*, and has unique 5′ EcoRI and 3′ HindIII sites into which each EcoRI/HindIII PCR-generated V_H gene segment (0.9 kb) was inserted.

DNA Transfection. L chain–secreting R4A cells were transfected by electroporation with plasmids encoding the wild-type or mutant R4A H chain V region genes (22). For each transfection reaction, 3×10^6 cell in 0.3 ml and 1–40 μg circular plasmid DNA, 1–40 μg, were used. After electroporation, cells were resuspended in 10 ml of selective (HX) medium, 1% L-glutamine (Mediatech, Herndon, VA), 1% penicillin-streptomycin (Mediatech), 1% nonessential amino acids, 0.003% fungizone, 0.002% gentamicin (all from GIBCO BRL, Gaithersburg, MD), 10 mM Hepes, pH 7.4, 0.15 mg/ml hypoxanthine, and 0.25 mg per ml xanthine (all from Sigma Chemical Co., St. Louis, MO), brought up to volume in IMDM (GIBCO BRL) supplemented with 20% FCS (Hyclone Labs., Logan, UT) and 10% supernatant from Con A–stimulated mouse spleen cells, and plated into 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ). After incubation at 37°C for 24–48 h, 6 μg/ml mycophenolic acid (Sigma Chemical Co.) was added to the culture medium. Surviving clones were screened for secretion of the H chain.

Purification of Antibodies. mAbs were purified from culture supernatants by elution (0.1 M glycine, pH3) from a protein G–Sepharose column on a fast protein liquid chromatography apparatus (Pharmacia, Piscataway, NJ).

Quantitation of Antibodies. 96-well microtiter ELISA plates (Becton Dickinson) were coated with a 1:500 dilution of goat anti-mouse IgG2b (Fisher Scientific Co., Pittsburgh, PA) for either 1 h at 37°C or overnight at 4°C. Wells were blocked with 2% BSA in PBS, washed 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 twofold dilutions of a commercially purified IgG2b standard. After washing, wells were incubated with a 1:1,000 dilution of alkaline phosphatase–linked goat anti-mouse IgG2b (Fisher Scientific) for 1 h and finally developed with alkaline phosphatase substrate (*p*-nitrophenyl tablets phosphate, Sigma Chemical Co.). The reaction was monitored at 405 nm.

k-γ2b ELISA. The k-γ2b ELISA was similar to the Cγ2b ELISA protocol described above but with the following modifications: (*a*) goat anti-mouse κ chain antibody (Fisher Scientific) was diluted 1:500 in PBS and adsorbed to a 96-well microtiter plate for 2 h at 37°C; and (*b*) purified antibodies were normalized to 5 μg/ml in blocking solution and added after block at 37°C for 1 h. Alkaline phosphatase–conjugated goat anti-mouse IgG2b was added for 1 h and the assay was developed with phosphatase substrate.

Anti-idiotypic ELISA. A monoclonal IgG1 anti-idiotypic reagent 9B4 that reacts with S107 V11–encoded H chains was generated by Dan Lustgarten (Albert Einstein College of Medicine). The ELISA was performed as follows. An ELISA plate was coated with 1 μg antibody at 37°C for 1–2 h. It was blocked, washed, and incubated with a 1:100 dilution in PBS of 9B4 cell supernatant, followed by an alkaline phosphatase–linked, goat anti-mouse IgG1 (Fisher Scientific) and developed in a standard manner.

ELISAs for Antigenic Specificity. The dsDNA ELISA used is a modification of the assay of Miller et al. (23). Round-bottom polyvinyl plates (Becton Dickinson) were coated with poly-L-lysine

(1 μg/well) for 2 h at 37°C. Calf thymus DNA was dissolved in 1× SSC at 4°C and filtered through a 0.45-M nitrocellulose Millex syringe filter (Millipore Corp.) to obtain dsDNA. 100 μl of dsDNA at an OD 260 of 0.75 was added to each well. The plates were blocked with 2% BSA/PBS for 1 h at 37°C and then incubated with 100 μl of purified antibody at 5 μg/ml for 1 h at 37°C. Anti-γ2b antibody diluted 1:1,000 was added for 1 h at room temperature followed by substrate. To prepare ssDNA, calf thymus DNA was dissolved in 1× SSC, boiled for 15 min, and put on ice. The ssDNA was also plated on poly-L-lysine–coated plates.

To measure RNA binding, microtiter plates were coated with 1 μg tRNA for 90 min at 37°C. Purified protein was tested at 5 μg/ml.

PC ELISA. The ELISA for PC binding was performed as described above with the following modifications: (*a*) the ELISA plates were coated with PC-KLH at 50 μg/ml for 2 h at 37°C; and (*b*) mAbs were normalized to 10 μg/ml.

dsDNA Millipore Filter Inhibition Assay. Unlabeled calf thymus dsDNA (Calbiochem-Novabiochem Corp., La Jolla, CA) in PBS was plated at various dilutions into each well of a Milliliter-HA 96-well filtration plate (Millipore Corp.). Purified antibody at a concentration of 5 μg/ml was preincubated with 10 μl of ^{32}P-labeled dsDNA at 1,500 cpm/μl for 1 h and then added to each well. The plate was incubated for an additional hour at 37°C. The samples were filtered through the plate and washed four to five times with 1× SSC on a 96-well vacuum filtration apparatus (Millipore Corp.). The wells were punched out and counted.

In Vivo Studies of Renal Pathology. 2 wk after pristane priming, 6–8-wk-old SCID mice were injected, intraperitoneally, with 10^7 cells. Five mice in each group were examined every week for proteinuria and serum levels of IgG2b. Proteinuria was examined by multistix (Miles Inc., Elkhart, IN). These mice were killed 1–2 wk after ascites and/or tumors developed. All mice used in this study had equivalent titers of IgG2b in their serum by ELISA (see above).

One kidney from each animal was fixed in 10% formalin and embedded in paraffin. Depositions of IgG antibodies in glomeruli were studied in 4-μm kidney sections which were deparaffinized, rehydrated, blocked with 2% BSA, stained with biotinylated goat anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA), then washed and developed with BCIP (5-Bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt) and NBT (Nitroblue Tetrazolium Chloride) substrates (GIBCO BRL). The stained sections were viewed with a Zeiss fluorescence microscope.

Results

Generation and Characterization of the R4A Mutants. R4A is a murine anti-dsDNA antibody. The H chain is encoded by S107 V11 and J_H4 gene segments, and an unknown D gene segment; the L chain by a member of the $V_\kappa1$ gene family and $J_\kappa1$. The R4A H chain was subjected to site-directed mutagenesis to study the effects of amino acid replacements on antibody affinity for dsDNA and/or fine specificity and pathogenicity. Mutant R4A H chain genes in a construct with the *gpt* gene were generated and transfected into an R4A H chain loss variant. The H chain V region sequence of the antibodies described in this report are given in Table 1. Three mutants 52, 85, and 95 were generated with single amino acid substitutions that alter the charge of the V region. 52 has a loss of a positively charged arginine at residue 52 in CDR2, 95 has a negatively charged residue removed from CDR3, and 85 has a loss of a negatively charged residue in FR3. The other mutants have multiple amino acid substitutions. Whereas most were designed to test the importance of arginine in dsDNA binding, some arose serendipitously. In 8382b and 9636, arginine was removed from FR3 or CDR3, respectively. In the three remaining mutants, 52b3, 52b6, and 6634, additional substitutions were made in charged amino acid residues. Arginine was removed from FR3 of 52b3. This same arginine was removed in 6634, but an arginine was reinserted into FR3 closer to CDR3. In 52b6 a lysine, also a positively charged amino acid, was removed from CDR2.

To confirm that the antibodies secreted by the transfectants show proper association of H and L chains, the wild-type and mutant antibodies were assayed in two ways. The first method utilizes an ELISA assaying for the coexpression of both H and L chain determinants on a single antibody molecule (Fig. 1). All antibodies displayed associated H and L chains, demonstrating that none of the mutations interfere with the assembly of H and L chains. The second assay tests for S107 V11 idiotype expression. The reactivity of the mutated antibodies was compared to that of the wild-type R4A molecule. All the mutants tested are recognized by the 9B4 anti-idiotype although three have slightly diminished reactivity (Fig. 2). We conclude that the H and L chains of all the transfected antibodies associate and that the H chain retains idiotypic expression.

We utilized a dsDNA ELISA to measure the relative binding of the wild-type and mutant antibodies to dsDNA (Fig. 3). The wild-type R4A shows midrange binding; there are mutants with no detectable dsDNA binding and mutants with markedly enhanced dsDNA binding. An inhibition assay was performed on antibody 52b3 with the highest relative binding for dsDNA in order to quantitate its change in apparent affinity (data not shown). From these data and using a formula derived by Nieto et al. (24) for measuring the apparent affinity of antibodies to antigens containing multiple repeated antigenic epitopes, one can calculate an apparent affinity (aKa) for 52b3 of $\sim 1.77 \times 10^{10}$ compared to $1–2 \times 10^9$ for the parental antibody. The mutants, therefore, range from no appreciable binding to dsDNA to a 10-fold increase in dsDNA binding.

To see if any of the mutations resulted in new antigenic specificities, the antibodies were tested for binding on a panel of structurally related antigens: ssDNA, RNA, and PC. The results of the ssDNA ELISA demonstrate that the wild-type R4A antibody does not bind single-stranded DNA (ssDNA) (Fig. 4 *a*). 52b3, which displays the most marked increase in affinity for dsDNA, also shows reactivity to ssDNA. Mutant 6634 likewise shows increased ssDNA and dsDNA binding on ELISA. Alterations in dsDNA and ssDNA binding are not always coordinate; mutant 52 demonstrates increased dsDNA reactivity but displays no acquisition of ssDNA reactivity.

The tRNA ELISA shows that neither the wild-type R4A antibody nor any mutant has specificity for this antigen (Fig. 4 *b*). The wild-type R4A antibody displays little reactivity to PC. Three of the mutants, however, seem to have gained reactivity to PC (Fig. 4 *c*). Mutant 52b3, which demonstrates

Table 1. *R4A Wild-type and Mutant H Chain Sequences*

	10	20	30	40	50
52b3	----------	----------	----------	----------	----------
6634	----------	----------	----------	----------	----------
52	----------	----------	----------	----------	----------
8382b	----------	----------	----------	----------	----------
85	----------	----------	----------	----------	----------
R4A	EVKLVESGGG	LVQPGGSLRL	SCATSGFTFT	**DYYMS**WVRQP	PGKALEWLG**F**
52b6	----------	----------	----------	----------	----------
9636	----M-----	----------	----------	----------	----------
95	----------	----------	----------	----------	----------

	60	70	80	90
52b3	--------Q-	----------	------A-S	----------
6634	--------G-	----------	------A-S-	--R-------
52	-S--------	----------	----------	----------
8382b	----------	----------	------A-S-	----------
85	----------	----------	----------	Q---------
R4A	**I RNKANGYTT**	T I SRDNSQS I	LYLQMNTLRA	EDSATYYCAR
52b6	---A------	----------	------A-S-	----------
9636	----------	----------	------A---	----------
95	----------	----------	----------	----------

	101	113
52b3	----------	--------
6634	----------	--------
52	----------	--------
8382b	----------	--------
85	----------	--------
R4A	**DR I PMDY**WGQ	GTSVTVSS
52b6	----------	--------
9636	-S--------	--------
95	G---------	--------

Antibodies are ordered from highest DNA binding (*top*) to no DNA binding (*bottom*). CDRs are denoted by bold letters and are underlined.

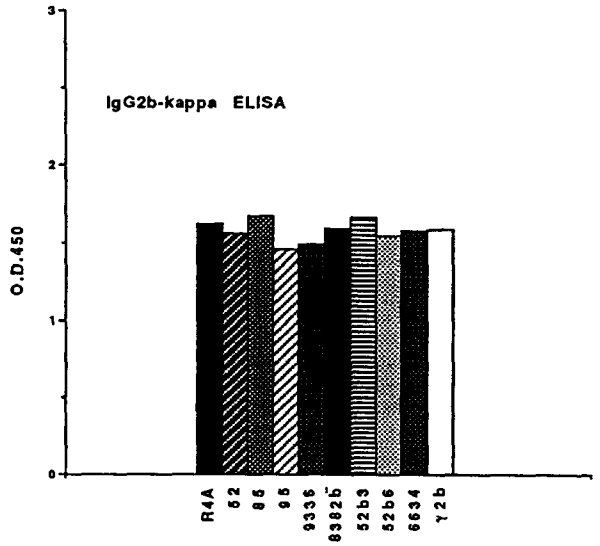

Figure 1. Association of γ2b H chain with κ L chains. Anti-κ chain antibody was adsorbed to microtiter plates. Purified protein was added, followed by an enzyme-linked anti-γ2b antibody. Absorbance at OD 405 was determined. The data in this assay are representative of several assays.

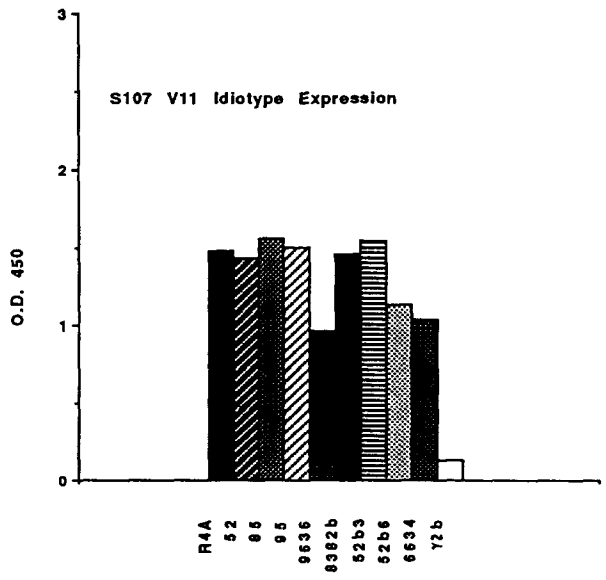

Figure 2. Expression of an S107 V11 determinant. Purified protein was adsorbed to microtiter plates. An IgG1 antibody specific for the S107 V11 gene product was added, followed by an enzyme-linked anti-γ1 antibody. Absorbance at OD 405 was determined. The data in this assay are representative of several assays.

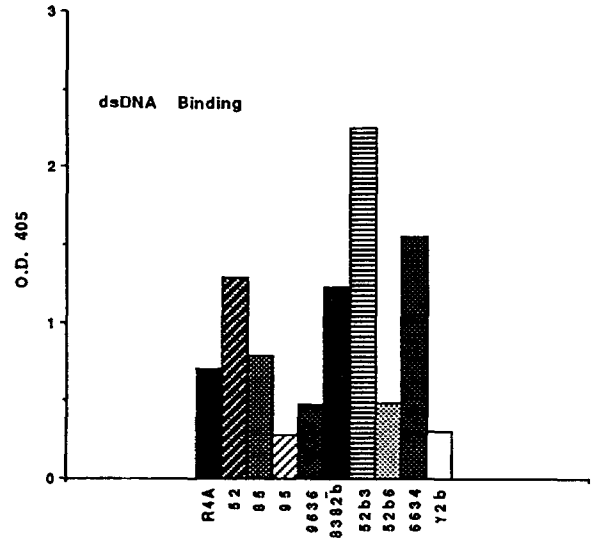

Figure 3. Binding to dsDNA. Binding to dsDNA was determined using an ELISA that measures binding to dsDNA bound to poly-L-lysine coated wells. Data in this assay are representative of several assays.

increased binding activity to dsDNA and ssDNA, displays the highest PC binding. Mutants 6634 and 85, which both bind dsDNA slightly better than the wild-type antibody, also display PC binding.

Three antibodies were assayed for pathogenicity, the parental R4A, 95, which has no detectable dsDNA binding, and 52b3, which displays the greatest increase in dsDNA binding. SCID mice were injected intraperitoneally with R4A cells or cells from the mutant lines, and mice displaying equivalent titers of IgG2b antibody in serum were used in this study (μg/ml R4A: 20, 39, 42, 61; 95: 8, 13, 18, 42, 75; and 52b3: 3, 16, 16, 18, 50). All mice carrying an R4A or 52b3 tumor developed significant proteinuria. The mutant 95 did not cause proteinuria in any mice. As shown in Fig. 5, immunohistochemical analysis demonstrated that R4A antibody is deposited in glomeruli in all mice. Antibody 95 is not sequestered in the kidney in any mice, consistent with its loss of DNA binding. Most surprisingly, all mice producing the 52b3 antibody showed some glomerular deposition of IgG2b but a more striking deposition of IgG2b in renal tubules. Thus the change in affinity demonstrated by the antibody also led to a change in renal localization.

Discussion

We have generated in vitro a genealogy of antibodies derived from the R4A parental cell line. The R4A line binds dsDNA and is deposited in renal glomeruli. Changes in specificity, relative affinity, and pathogenicity were measured. The following amino acids were targeted for substitution. (*a*) Arginine residues were replaced with serine in CDR2, CDR3, and FR3 since arginine is the amino acid most closely associated with dsDNA binding; (*b*) lysine was targeted since it is also a basic amino acid and is present in the nucleotide binding sites of an anti-ssDNA antibody and other DNA

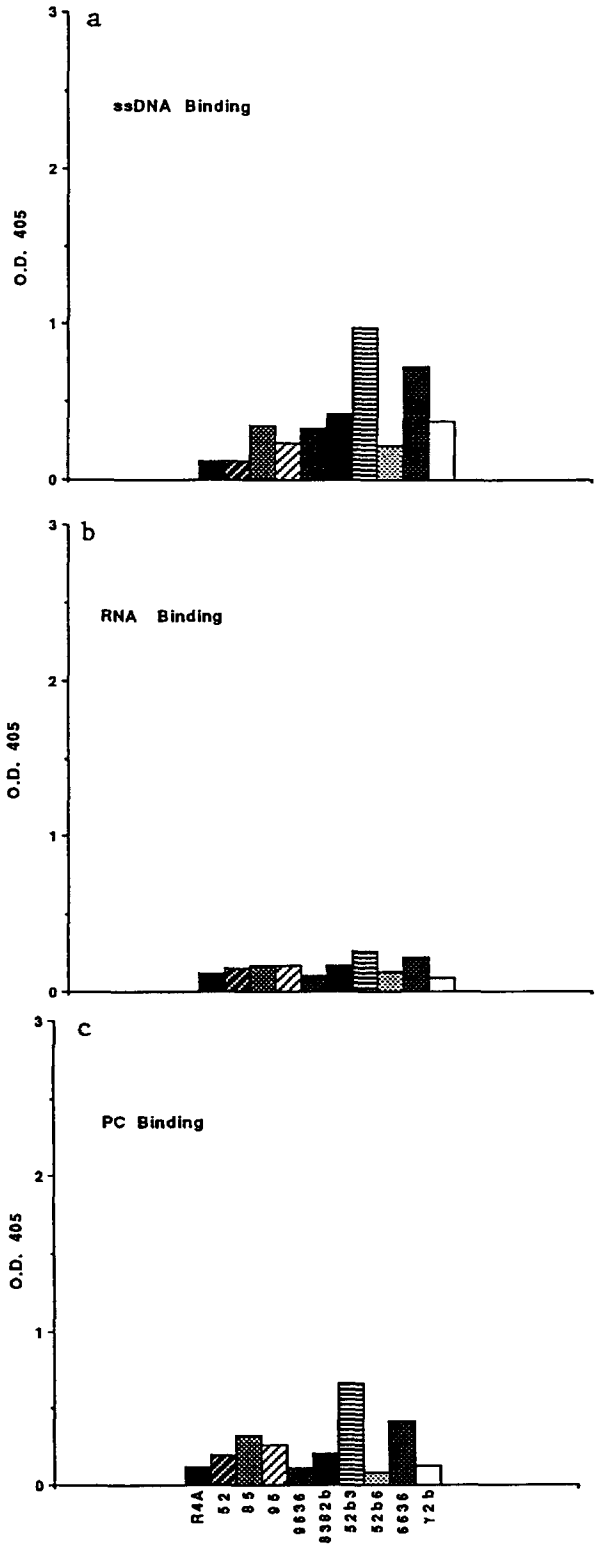

Figure 4. Binding to dsDNA-related antigens. Binding to ssDNA, RNA and PC was performed by ELISA as described in Materials and Methods. All assays are representative of several assays.

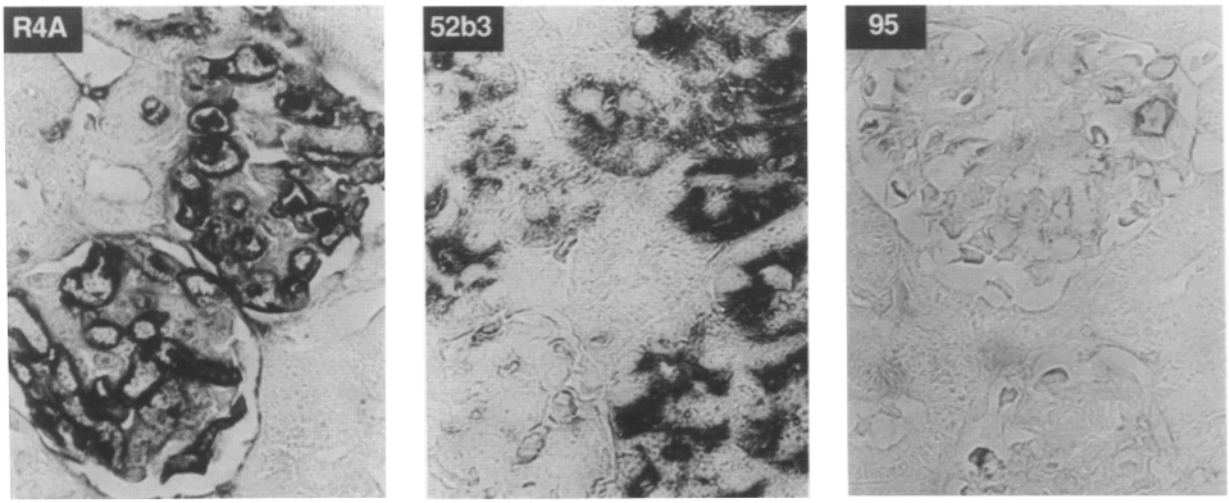

Figure 5. Kidney deposition of R4A and its mutants. Kidney sections from 6–8-wk-old SCID mice given receiving R4A or mutant cells were stained with biotinylated anti-IgG. Immune deposits were visualized in glomeruli for R4A and preferentially in tubules for the 52b3 mutant. No IgG deposition was observed for the 95 mutant.

binding proteins (12, 25); and last, (*c*) the acidic amino acids, glutamic acid and aspartic acid, were substituted in FR3 and CDR3, respectively. If charge alone is important in the interaction with DNA, then the presence of aspartic acid and glutamic acid should inhibit interactions with DNA.

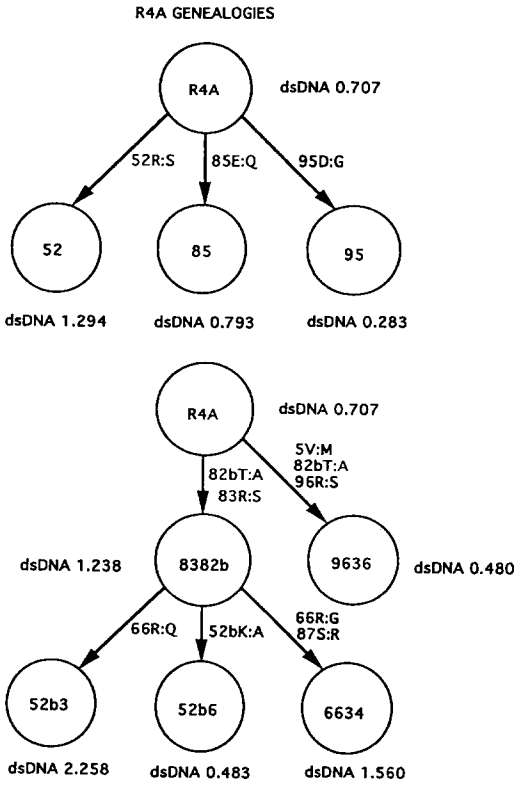

Figure 6. Genealogies of R4A mutants. One genealogy shows variants with single amino acid substitutions. The second genealogy shows variants with two- to three-amino acid substitutions. The amino acid substitutions and the dsDNA binding are shown for each variant.

Three mutants with single amino acid changes were isolated (Fig. 6). Mutant 52 has one amino acid substitution, 52 arginine to Ser in CDR2. It demonstrates higher binding than R4A to dsDNA, showing that an arginine may be lost within a CDR without a resulting decrease in dsDNA binding. The second mutant in this class, 85, has an 85 glutamic acid to glutamine replacement in FR3 and shows a modest increase in dsDNA binding, demonstrating that FR substitutions can alter DNA binding. Mutant 95, with a 95 aspartic acid to glycine replacement in CDR3 is notable for its loss of binding to dsDNA. The fact that the loss of a negatively charged amino acid leads to decreased dsDNA binding is surprising as positively charged amino acids have been implicated in DNA binding. Even more significant is the finding that this antibody, which is more cationic than the parental R4A, is no longer sequestered in glomeruli. Cationic antibodies are thought to be preferentially sequestered in glomeruli because of their interaction with anionic basement membrane. This suggests that both antigenic specificity and charge are important in generating pathogenicity.

The other mutants with multiple amino acid changes can be organized into an artificially generated B cell genealogy based on shared mutations (Fig. 6). Some structural conclusions can be drawn from the analysis of these more complex mutants. First, CDRs contain amino acids which are critical to the antigenic specificity of R4A. As expected, basic amino acids residues 52b and 96 can contribute to DNA binding. Residues 66, 82b, and 83 in FR3 of the H chain can have a significant influence on the specificity and affinity of an anti-DNA antibody. A substitution in residue 66, like residue 85, is associated with both a change in affinity and the acquisition of new fine specificities.

It is interesting to note that two of the replacement mutations presented in this paper represent substitutions of invariant residues (26). 52b lysine and 83 arginine, are mutated to alanine and serine, respectively. All of the proteins which

bear these mutations show the characteristics of an intact protein by the κ-γ2b ELISA and the S107 V11 idiotypic ELISA. Conformational information regarding these antibodies will depend on obtaining crystallographic data, but the demonstration that they are secreted in culture, show normal association of H and L chains, and maintain an idiotypic marker argues against the notion that the presence of these residues is required either for transport and secretion or for maintaining gross conformation. Similar results were obtained by Sharon (27) when she mutated an invariant residue of the H chain, 36 tyrosine. It is not clear why the invariant residues are conserved but one tempting speculation is that the role of the evolutionarily maintained invariant residues lies in the need to preserve idiotypic regulatory epitopes.

The 52b3 antibody which is mutated at three residues in FR3 of the H chain has a dramatic alteration in renal pathology. Whereas it continues to show some glomerular deposition, it is now deposited to a far greater extent in tubules, suggesting that the change affinity for dsDNA is also associated with the acquisition of a novel antigenic cross reactivity.

A major conclusion from the study of these mutant antibodies is that one or a few amino acid differences can alter antigen binding. This has been shown previously with an in vitro–generated mutant of a mouse myeloma cell line (28) and with B cell genealogies obtained by somatic cell fusion (29, 30). Whereas expressed antibody molecules often display many amino acid differences from the germline template, only one or two of these may be significant in changing antigenic specificity or affinity. Antibodies with only one or a few mutations would not be identified as antigen-selected antibodies in a mutational analysis where numbers of substitutions or R/S ratios are scored yet may display marked differences in antigen binding. This demonstrates the limitations of mutational analyses that do not also monitor affinity and specificity. Furthermore, the results of some of the mutations reported there suggest that we cannot yet accurately predict those residues that are critical to DNA binding.

A second conclusion is that FR determinants do not serve a purely scaffolding function; they can influence antigenic fine specificity and affinity. Others have also shown that amino acid replacements in FR can affect antigen binding (31–33). Behar et al. (5) described a panel of anti-dsDNA antibodies derived from an autoimmune mouse in which some of the antibodies with high affinity dsDNA binding have a high R/S ratio in the FRs. Perhaps the replacement mutations in FR helped create dsDNA binding specificity either by direct interaction with antigen or through distal conformational effects.

The most dramatic finding of this study is that a very few amino acid substitutions can completely abrogate pathogenicity or alter the in vivo target of a pathogenic autoantibody. The observation that two closely related antibodies that differ in affinity for dsDNA binding R4A and 52b3 are discordant with respect to localization of renal deposition raises the question of whether DNA binding is central or incidental to the pathogenicity of these antibodies. It seems unlikely that DNA is differentially present in glomeruli where it is recognized by R4A and tubules where it bound by 52b3, or even that DNA–anti-DNA complexes are differentially harbored in these sites. More likely, anti-DNA antibodies cross-react with renal antigens, often with glomerular antigens but occasionally with tubular antigens. Others have demonstrated direct binding of anti-DNA antibodies to glomerular or cell surface antigens (14, 15), also suggesting that DNA binding may be an epiphenomenon of lupus autoantibodies. The as yet undefined cross-reactivities of anti-DNA antibodies may well represent their important autoantigenic specificities in the pathogenesis of SLE.

The authors wish to thank Matthew Scharff for useful discussions, Millie Borgas and Mary Martin for preparation of the manuscript, and Czeslowa Kowal for the computer graphics.

This work was supported by National Institutes of Health grants AR-32371 and AI-33184. J. B. Katz was supported by training grant T32GM7288. W. Limpanasithikul is supported by a scholarship from Chulalongorn University, Thailand. Oligonucleotides were generated at the Oligonucleotide Synthesis Facility of the Albert Einstein College of Medicine supported by National Cancer Institute grant CA-13330.

Address correspondence to Dr. Betty Diamond, Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461-1602.

Received for publication 2 February 1994 and in revised form 24 May 1994.

References

1. Pearson, L., and R.W. Lightfoot, Jr. 1981. Correlation of DNA-anti-DNA association rates with clinical activity in systemic lupus erythematosus. *J. Immunol.* 126:16.
2. Winfield, J.B., I. Faiferman, and D. Koffler. 1977. Activity of anti-DNA antibodies in serum and IgG glomerular eluates in patients with systemic lupus erythematosus: association of high avidity anti-native DNA antibody with glomerulonephritis. *J. Clin. Invest.* 59:90.
3. Marion, T.N., D.M. Tillman, and N. Jou. 1990. Interclonal and intraclonal diversity among anti-DNA antibodies from an (NZBXNZW) F1 mouse. *J. Immunol.* 145:2322.
4. Shlomchik, M., M. Mascelli, H. Shan, M.Z. Radic, D. Pisetsky,

A. Marshak-Rothstein, and M. Weigert. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J. Exp. Med.* 171:265.
5. Behar, S.M., D.L. Lustgarten, S. Corbet, and M.D. Scharff. 1991. Characterization of somatically mutated S107 V_H11 encoded anti-DNA autoantibodies derived from autoimmune (NZBXNZW) F1 mice. *J. Exp. Med.* 173:731.
6. O'Keefe, T.L., S. Bandyopadhyay, S.K. Datta, and T. Imanishi-Kari. 1990. V region sequences of an idiotypically connected family of pathogenic anti-DNA autoantibodies. *J. Immunol.* 144:4275.
7. Shlomchik, M.J., A. Marshak-Rothstein, C.B. Wolfowicz, T.H. Rothstein, and M.G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature (Lond.).* 328:805.
8. van Es, J.H., F.H.J. Gmelig-Meyling, W.R.M. van de Akker, H. Aanstoot, R.H.W.M. Derksen, and T. Logtenberg. 1991. Somatic mutations in the variable regions of a human IgG anti-double-stranded DNA autoantibody suggest a role for antigen in the induction of systemic lupus erythematosus. *J. Exp. Med.* 173:461.
9. Davidson, A., A. Manheimer-Lory, C. Aranow, R. Peterson, N. Hannigan, and B. Diamond. 1990. Molecular characterization of somatically mutated anti-DNA antibody bearing two systemic lupus erythematosus-related idiotypes. *J. Clin. Invest.* 85:1401.
10. Behar, S.M., and M.D. Scharff. 1988. Somatic diversification of the S107 (T15) V_H11 germline gene that encodes the heavy chain variable region of antibodies to double stranded DNA in (NZB/NZW) F1 mice. *Proc. Natl. Acad. Sci. USA.* 85:3970.
11. Seeman, N.C., J.M. Rosenberg, and A. Rich. 1976. Sequence specific recognition of double helical nucleic acids by proteins. *Proc. Natl. Acad. Sci. USA.* 73:804.
12. Herron, J.N., X.M. He, D.W. Ballard, P.R. Blier, P.E. Pace, A.L.M. Bothwell, E.W. Voss, Jr., and A.B. Edmundson. 1991. An autoantibody to single-stranded DNA: comparison of the three-dimensional structures of the unliganded Fab and a deoxynucleotide Fab complex. *Proteins.* 11:159.
13. Ebling, F.M., and B.H. Hahn. 1980. Restricted sub-populations of DNA antibodies in kidneys of mice with systemic lupus: comparison of antibodies in serum and renal eluates. *Arthritis Rheum.* 23:392.
14. Vlahakos, D.V., M.H. Foster, S. Adams, M. Katz, A.A. Ucci, K.J. Barrett, S.K. Datta, and M.P. Madaio. 1992. Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites. *Kidney Int.* 41:1690.
15. Madaio, M.P., J. Carlson, J. Cataldo, A. Ucci, P. Migliorini, and O. Pankewycz. 1987. Murine monoclonal anti-DNA antibodies bind directly to glomerular antigens and form immune deposits. *J. Immunol.* 138:2883.
16. Ohnishi, K., F.M. Ebling, B. Mitchell, R.R. Singh, B.H. Hahn, and B.P. Tsao. 1994. Comparison of pathogenic and nonpathogenic murine antibodies to DNA: antigen binding and structural characteristics. *Int. Immunol.* 6:817.
17. Shefner, R., G. Kleiner, A. Turken, L. Papazian, and B. Diamond. 1991. A novel class of anti-DNA antibodies identified in BALB/c mice. *J. Exp. Med.* 173:287.
18. Baumal, R., B.K. Birshtein, P. Coffino, and M.D. Sharff. 1973. Mutations in immunoglobulin producing mouse myeloma cells. *Science (Wash. DC).* 182:164.
19. Manser, T., and M.L. Gefter. 1984. Isolation of hybridomas expressing specific heavy chain gene segments by using a screening technique that detects mRNA sequences in whole cell lysates. *Proc. Natl. Acad. Sci. USA.* 81:2470.
20. Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 77:51.
21. Sharon, J., M.L. Gefter, L.J. Wysocki, and M.N. Margolies. 1989. The recurrent somatic mutations in mouse antibodies to *p*-azophenylarsonate increase affinity for hapten. *J. Immunol.* 142:596.
22. Sharon, J. 1990. Structural correlates of high antibody affinity: three engineered amino acid substitutions can increase the affinity of an anti-*p*-agophemylarsonate antibody 200-fold. *Proc. Natl. Acad. Sci. USA.* 87:4814.
23. Miller, T.E., R.G. Lahita, V.J. Zarro, J. MacWilliam, and D. Koffler. 1981. Clinical significance of anti-dsDNA antibodies detected by a solid phase enzyme immunoassay. *Arthritis Rheum.* 24:602.
24. Nieto, A., A. Gaya, M. Jansa, C. Moreno, and J. Vives. 1984. Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. *Mol. Immunol.* 21:537.
25. Takeda, Y., J.G. Kim, C. Caday, E. Steers, Jr., D.H. Ohlendorf, W.F. Anderson, and B.W. Matthews. 1986. Different interaction used by Cro repressor in specific and nonspecific DNA binding. *J. Biol. Chem.* 261:8608.
26. Kabat, E.A., T.T. Wu, M. Reid-Miller, H.M. Perry, and K.S. Gottesman. 1991. Sequences of Proteins of Immunological Interest. U.S. Department of Health and Human Services. Bethesda, MD.
27. Sharon, J. 1988. The invariant tryptophan in an H chain V region is not essential to antibody binding. *J. Immunol.* 140:2666.
28. Diamond, B., and M.D. Scharff. 1984. Somatic mutation of the T15 heavy chain gives rise to an antibody with autospecificity. *Proc. Natl. Acad. Sci. USA.* 81:5841.
29. Krishnan, M.R., and T.N. Marion. 1993. Structural similarity of antibody variable regions from immune and autoimmune anti-DNA antibodies. *J. Immunol.* 150:4948.
30. Berek, C., Griffin, G.M., and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature (Lond.).* 316:412.
31. Tramanto, A., C. Clothia, and A.M. Lesk. 1990. Framework residue is a major determinant of the position and conformation of the second hypervariable region in the V_H domains of immunoglobulins. *J. Mol. Biol.* 215:175.
32. Panka, D.J., M. Mudgett-Hunter, D.R. Parks, L.L. Peterson, L.A. Herzenberg, E. Haber, and D.R. Margolies. 1988. Variable region framework differences result in decreased or increased affinity of variant anti-digoxin antibodies. *Proc. Natl. Acad. Sci. USA.* 85:3080.
33. Chien, N.C., V.A. Roberts, A.M. Giusti, M.D. Sharff, and E.D. Getzoff. 1989. Significant structural and functional change of an antigen binding site by a distant amino acid substitution: proposal of a structural mechanism. *Proc. Natl. Acad. Sci. USA.* 86:5532.