ANTI-DNA ANTIBODIES FROM AUTOIMMUNE MICE ARISE BY CLONAL EXPANSION AND SOMATIC MUTATION

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Systemic autoimmune diseases, such as systemic lupus erythematosus (SLE)[1] in humans, are characterized by abnormal immune recognition and destruction of multiple organs and tissues. The immune defect appears to involve both T and B cells, although the nature of the primary disturbance(s) that ultimately leads to loss of immunologic self-tolerance is unknown. Many of the systemic autoimmune diseases are accompanied by serological evidence of autoimmunity in the form of circulating antibodies to tissue and blood macromolecules (1–3). The presence of various autoantibodies is specific for disease, and certain autoantibodies are diagnostic of particular autoimmune syndromes (2, 3). The titers of some types of autoantibodies, for example anti-IgG (rheumatoid factors [RF]) in rheumatoid arthritis (4, 5) and antibodies to dsDNA in SLE (1, 6), correlate with disease activity. Anti-DNA in particular have been implicated in pathogenesis by direct evidence of DNA-anti-DNA complexes in diseased kidney (6, 7), as well as evidence that injected anti-DNA is nephritogenic (8, 9). Understanding what causes the production of autoantibodies should provide insight into the cause of the disease itself.

We have exploited the MRL/*lpr* mouse model for SLE to study the etiology of humoral autoimmunity. Disease in this strain is dependent on the recessive *lpr* gene and unknown background genes (10, 11). Although the MRL/*lpr* syndrome is different from human SLE in that the *lpr* gene causes proliferation of an unusual subset of T cells (12), MRL/*lpr* disease is strikingly similar in a number of aspects to human disease. These mice develop multi-system autoimmunity, including a lupus-like nephritis (11), arthritis (13) and antinuclear antibodies, antibodies to ribonucleoproteins (14), and antibodies to ssDNA and dsDNA (15).

The study of somatic cell hybrids formed with unmanipulated spleen cells from MRL/*lpr* mice has recently allowed us to address the question: is the stimulus for

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[1] *Abbreviations used in this paper:* CDR, complementarity determining region; R, replacement; RF, rheumatoid factor; SLE, systemic lupus erythematosus.

production of autoantibodies nonspecific polyclonal B cell activation or specific (auto)antigen-driven B cell activation? The advantage of the cell hybridization approach is that it captures both specifically and nonspecifically activated spleen cells; antigen-activated B cells captured in this way have been shown to accurately reflect the humoral immune response (16), and panels of hybrids from polyclonally activated B cells are indeed polyclonal (17, 18). Recently, we showed that the V gene sequences and rearrangements of spontaneous monoclonal RFs from MRL/*lpr* mice were the product of specific B cell activation mediated via the antigen receptor (19).

We now present a similar analysis of a classical SLE autoantibody: anti-DNA. This analysis shows that anti-DNA, like RFs, are oligoclonal and are the result of specific receptor-driven stimulation, providing strong evidence that this is the case for MRL/*lpr* autoimmunity in general. Furthermore, knowledge of both V region sequences and antibody specificities shows that: (*a*) somatic mutations can give rise to the disease-specific anti-dsDNA and that such mutations are selected for; (*b*) arginine residues play an important role in determining specificity; and (*c*) antiidiotypes that recognize the majority of anti-DNA are probably not specific for any one family of V regions. These results have important implications for both etiology and therapy of autoimmunity.

Materials and Methods

Antibodies. All mAbs were derived from fusions of spleen cells from MRL/*lpr* mice (obtained from The Jackson Laboratory, Bar Harbor, ME) with myeloma cell lines. The generation and initial characterization of antibodies from mouse 1 and 2 were described previously (20, 21). Hybridomas from mouse 3 (28 wk of age) were prepared according to the procedure of Marshak-Rothstein et al. (22). Half of the spleen cells were fused with the cell line SP2/0, the other half with Ag8. Somatic cell hybrids from mice 4 and 5 were generated according to the technique of Oi and Herzenberg (23), using the myeloma cell line NS1 as the fusion partner. Mouse 4 was 14 wk old and mouse 5 was 21 wk old. Hybridoma supernatants were screened for antibodies to denatured calf thymus DNA by an ELISA, as described (24). The frequency of anti-DNA hybridomas was 0.19 and 0.06 for mouse 3 and 4, respectively. The frequency was not determined for the other mice. Cells from randomly selected positive wells were cloned twice by limiting dilution. The H and L chain isotypes of the anti-DNA mAbs were determined using subclass-specific reagents (Litton Bionetics, Charleston, SC).

Specificity Analysis. Tissue culture supernatants of each antibody were collected at mid-log phase of growth and stored at 4°C with 0.02% NaN_3. The concentration of antibody in the supernatants was determined by ELISA, using the following purified myeloma proteins as standards. FLOPC/21 (IgG3) was purchased from Sigma Chemical Co. (St. Louis, MO). The myeloma cell lines 7043 (IgG2a), 2413 (IgG2b), 7210 (IgG1), and 3741 (IgM) (25), were maintained in this laboratory and purified from ascites fluid by standard column chromatography methods.

A standard direct binding ELISA (26) was used to determine mAb binding to ssDNA. A novel assay was used to assess antibody binding to both ss- and dsDNA in solution (Radic, M. Z., and M. Weigert, manuscript in preparation). Briefly, salmon sperm DNA (Sigma Chemical Co.) was purified by treatment with protease and multiple phenol and chloroform extractions, followed by repeated precipitation in ethanol. It was then photobiotinylated using the reagent and protocol of Vector Laboratories, Inc. (Burlingame, CA). From this stock, dsDNA was prepared by treating with S1 nuclease, followed by Hae III digestion to yield flush ends. ssDNA was prepared by denaturing dsDNA at 90°C in 10 mM Tris (pH 7.2), 1 mM EDTA for 10 min before dilution in ice-cold PBS-BSA. Dilutions of antibody supernatants in PBS, containing 1% BSA, were incubated with biotinylated DNA (concentration, 2 μg/ml), for 3 h at 37°C. The incubation mixtures were then transferred to microtiter plates that were precoated with 5 μg/ml goat anti-mouse κ antibody (Fisher Biotech, Fair Lawn,

NJ). After a 2-h incubation at 37°C, the plates were washed twice with PBS, containing 0.05% Tween 20. Alkaline phosphatase-conjugated streptavidin (Fisher Biotech) was then added according to the recommended dilution, and incubated for 30 min at 37°C. The plates were again washed twice before the addition of a 1% paranitrophenyl phosphate solution in 50 mM $NaHCO_3$, 10 mM $MgCl_2$; pH 9.6. Relative binding affinities were determined by calculating the concentration of antibody that generated half-maximum absorbance at 405 nm for both denatured and native DNA. The data (Table II) is represented as the ratio of the half-maximal concentration of native dsDNA over ssDNA. To establish the validity of this assay, two anti-DNA mAbs, H241 and C11, were used as standards because their specificities for dsDNA and ssDNA have been well characterized. H241 recognizes both dsDNA and ssDNA (27), whereas C11 is specific just for ssDNA (24). In this assay, H241 bound to both forms of DNA. C11 bound to ssDNA and showed no significant binding to dsDNA, even at antibody concentrations 30-fold higher than the concentration to achieve half-maximal ssDNA binding.

Oligonucleotides. Oligonucleotides complementary to the 5' border of the mRNA encoding the C regions of the H and κ isotypes were synthesized on an oligonucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA). These were purified by electrophoresis through 20% polyacrylamide, 7 M urea gels, followed by absorption to Sep-Pak C18 cartridges (Waters Associates, Milford, MA), and elution in 20% acetonitrile. Sequences of oligonucleotides complementary to the μ, γ_3, and κ, and crosscomplementary to γ_1, γ_{2a} and γ_{2b} C regions were as previously reported (28, 29). The $J_\kappa 1$ probe (sequence 5'TGATTTCCAGCTTC-CTGCCTCCAC3'), was a gift from Dr. Andrew Caton (Wistar Institute, Philadelphia, PA).

RNA Isolation and Nucleotide Sequencing. The majority of the V regions were sequenced by synthesizing cDNA from poly(A)$^+$ RNA using oligonucleotide primers labeled with ^{32}P at the 5' end. cDNA was purified and sequenced by a rapid chemical degradation technique, as described by Shlomchik et al. (29). The V_H and V_κ regions of hybridomas DP7 and DP11 and the V_H regions of DP1 and 12 were sequenced directly from the poly(A)$^+$ RNA template by the oligonucleotide-primed dideoxy chain termination method according to Geliebter et al. (30). The nucleic acid sequences were compared with Ig sequences stored in the EMBL/GenBank Data Libraries.

Results

The Anti-DNA Response in MRL/lpr Mice Is Oligoclonal. The V_H and V_κ regions of 31 anti-DNA mAbs from five MRL/*lpr* mice were sequenced and the results are shown in Table I, which summarizes the V_H, D_H, J_H, V_κ, and J_κ segments used. The data show that for each mouse, most antibodies can be grouped into one or more sets, based on their use of the same or highly similar V_H and V_L genes. For instance, all antibodies from mouse 1 (set A), two from mouse 2 (set B), five from mouse 3 (set C), and five from mouse 4 (set D) are coded for by V_H and V_κ genes, which are 98–100% homologous. Mouse 5 is more complex, with three such sets (E, F, and G).

Use of the same V gene pairs among antibodies of individual mice, but rarely between mice, suggests that antibodies of a set are derived from a single B cell precursor. This possibility was borne out by more detailed analysis of the sequences of the third complementarity determining region (CDR3) of V_H and the rearranged V_H and V_κ alleles. Because of nucleotide addition (31) and deletion during VDJ joining, the nucleotide sequence in the CDR3 of the H chain is highly variable, even among antibodies with specificity for the same hapten (32; T. Manser, personal communication). Identity of CDR3 nucleotide sequence from unrelated B cells is very unlikely (see Appendix). As shown in Table I and Fig. 1, members of a set defined by identical V_H, D_H, and J_H segment usage had identical V_H CDR3 sequences as

TABLE I

Summary of Sequence Data of Anti-DNA-producing Hybridomas

Mouse	Cell	Clone	Isotype	V_κ group	J_κ	V_H family	D_H	J_H	CDR3
1	1A11	A	IgG2b	4	4	J558	SP 2-5,7	4	11
1	4H8	A	IgG2b	4	4	J558	SP 2-5,7	4	11
1	2F2	A	IgG2b	4	4	J558	SP 2-5,7	4	11
1	3H9	A	IgG2b	4	4	J558	SP 2-5,7	4	11
2	6O		IgG2a	9	1	Vgam3	SP 2/SP 2-7	4	11
2	6P		IgG1	12-13	2	ND	ND	ND	
2	6N	B	IgG2a	24	2	J558	SP 2/FL 16-1	2	9
2	6Q	B	IgG2a	24	2	J558	SP 2/FL 16-1	2	9
3	S7	C	IgG3	8	5	J558	Q 52	2	11
3	S41	C	IgG3	8	5	J558	Q 52	2	11
3	S54	C	IgG3	8	5	J558	Q 52	2	11
3	S57	C	IgG3	8	5	J558	Q 52	2	11
3	S204	C	ND	8	5	ND	ND	ND	ND
3	S106		IgG3	24	4	7183	SP 2-5,7,8	3	11
3	D23		IgM	2	1	J558	FL 16-2	3	8
3	D20		IgG2a	8	5	J558	FL 16-2	3	12
4	1E-81		IgG2a	1	2	J558	FL 16-1	3	9
4	3E12	D	IgG1	31*	2	7183	SP 2-5,7	1	10
4	6G6	D	IgG1	31*	2	7183	SP 2-5,7	1	10
4	3G9	D	IgG1	31*	2	7183	SP 2-5,7	1	10
4	2E3	D	IgG1	31*	2	7183	SP 2-5,7	1	10
4	6B8	D	IgG1	31*	2	7183	SP 2-5,7	1	10
5	DP1		IgM	1	2	S107	SP 2/SP 2-5,7,8	3	4
5	DP12		IgM	23	2	7183	SP 2-3,4,5,6,7	2	7
5	DP7	E	IgG3	9	1	J558	SP 2-8	3	6
5	DP11	E	IgG3	9	1	J558	SP 2-8	3	6
5	DP15	(E)	ND	9	1	ND	ND	ND	ND
5	DP17	F	IgG1	19	1	J558	FL 16-2	3	11
5	DP9	F	IgG1	19	1	J558	FL 16-2	3	11
5	DP13	G	IgG1	8	5	ND	ND	ND	ND
5	DP18	(G)	ND	8	5	ND	ND	ND	ND

Hybrids were assigned to clones according to the criteria outlined in the text. Clone names are not given for cells without siblings. Clone assignments of DP15 and DP17 are in parentheses to indicate they are based on κ chain sequence only; no V_H sequence was obtained. V_H and V_κ assignments to homology groups are made based on >80% sequence homology to known prototypes. The nomenclature for V_κ group numbers is as described by Potter et al. (39), and for V_H families by Brodeur and Riblet (33), except that Vgam3.8 is the name given to a family of V_H genes identified by Winter et al. (34). D_H designations are by homology to the closest known BALB/c gene (46). The CDR3 length is defined as the number of amino acids between the last amino acid encoded by V_H genes (two residues after the invariant cysteine) and the invariant tryptophan residue encoded by all J_Hs.

* This V_κ sequence does not meet the criteria for assignment to a known V_κ group (39). We therefore assign it to a new V_κ group, 31.

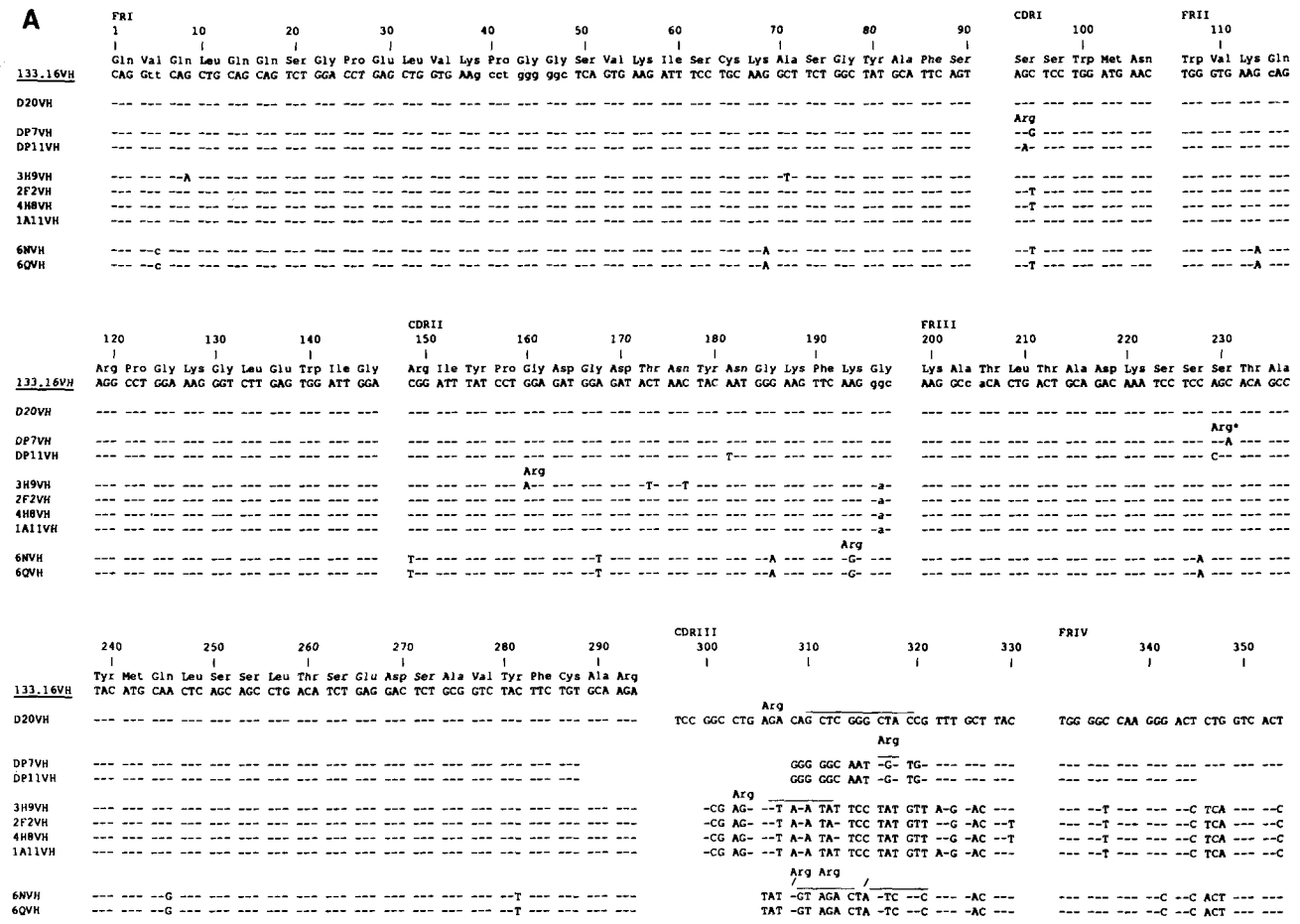
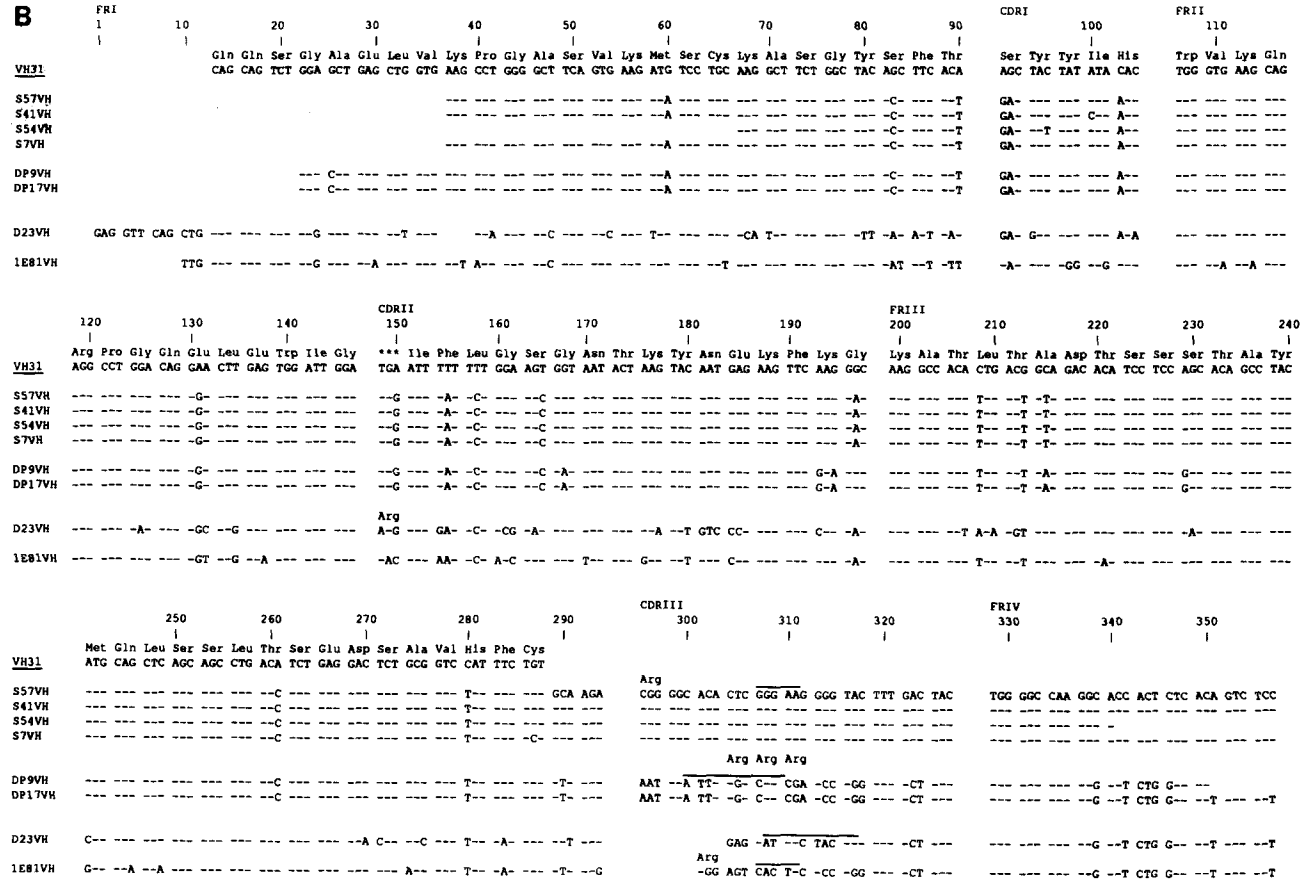


FIGURE 1. Legend on page 273.



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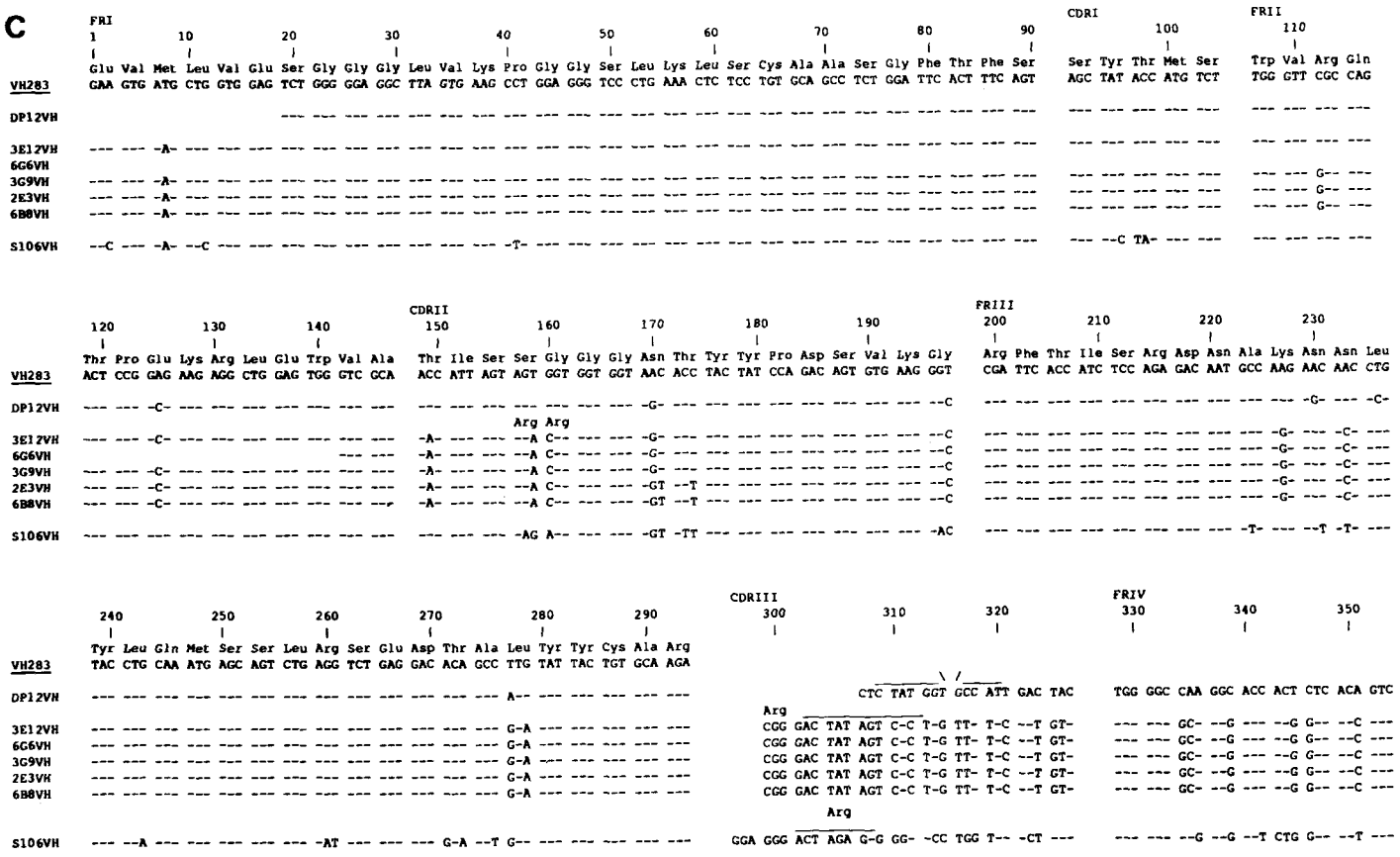


FIGURE I. Continued.

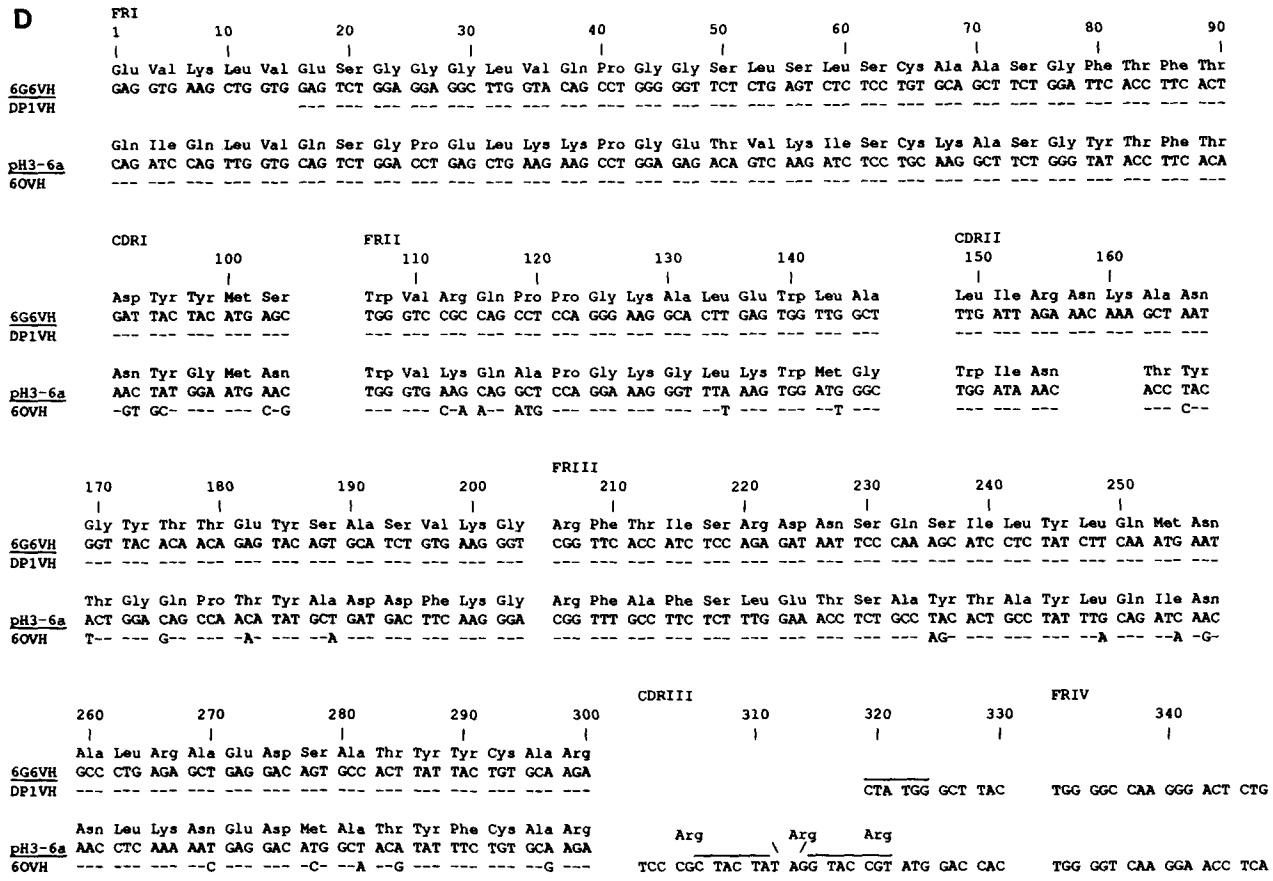


FIGURE 1. Continued.

well. This criterion alone makes it unlikely that members within a set are independently derived. Even using a conservative model system (the highly restricted response to *p*-azophenylarsonate) and conservative statistical assumptions, we find the chance of two unrelated B cells having identical CDR3 nucleotide sequences is 0.067 and the chance of three unrelated B cells sharing CDR3 sequence is 0.023 (see Appendix).

Related B cells can also be identified by the context of their unexpressed V_H and V_L alleles if these are not in the germline configuration. Identity of restriction fragment lengths at the unexpressed alleles of unrelated B cells is unlikely (see Appendix). Southern analysis of the H chain alleles of members in sets A, C, D, and E showed that in each case, the majority of hybrids in a putative clone had identical restriction fragment lengths at both alleles, whereas patterns differed between clones (data not shown). In the rest of the members of these clones and in clones B, F, and G, the expected fragment containing the unexpressed allele was absent, which we attribute to segregation of chromosomes after cell fusion. The restriction patterns at the κ alleles were uninformative, as all clones either had unrearranged alleles or multiple members that segregated the unexpressed allele.

In summary, the majority of the DNA antibodies in individual mice are members of one or a few clones (Table I), since each set meets at least two of the three criteria for clonal relatedness discussed above. The majority (50–100%) of the DNA antibodies of all five of the MRL/*lpr* mice are produced by just one or a few clones. These clones must, therefore, have undergone a significant amount of in vivo expansion. Given that hybridization immortalizes 1 of 10^4–10^5 of splenic B cells (16; our unpublished data), even two related hybrids represent clones on the order of 2×10^4 to 2×10^5 cells. This analysis also identified six hybridomas that could not be

FIGURE 1. Nucleotide sequences of H chain (V_H) regions of anti-DNA antibodies. The lettered subparts of the figure show homologous sets of sequences all belonging to the same V_H family, with the exception of *D*, which includes sequences from two V_H families. Sequences that are from clonally related hybridomas (see text) are grouped, followed by a space. Sequences in each subpart are compared with a single prototype (the names of which are underlined), with the exception of *D*, which uses two prototypes. Identities are indicated by dashes. The translation of the prototypes are given above the nucleotide sequence. Spaces are introduced to maximize alignment; a gap is introduced at the end of the V-encoded region (i.e., two codons after the invariant cys codon) to maximize homology with J_H. Spaces at 5′ ends of some sequences indicate that these regions have not been determined. Arginine codons in V_H CDR3 or that result from differences with the consensus or prototype sequence are designated with Arg. The Arg designating codons at position 77 of the V_H of DP7 (AGA) and DP11 (CGC) are marked by an asterisk to indicate that both codons encode arginine. The start of the framework (FR) and CDR (44), are separated by vertical spaces, and the top line of each sequence group gives the name of each subregion (e.g., CDR1 or FR2). A bar has been placed over nucleotides with homology to a known BALB/c germline D gene. Two bars over the same sequence indicate a possible fusion of two D gene segments. The direction of the arrow indicates whether sense or antisense strand of the D gene is translated (see text for details). The sources of prototype sequences are: (*A*) VH133.16 from a mAb from a C57Bl/6 mouse (43). Gaps in the reported sequence of VH133.16 have been filled with the consensus of all members in the group. These regions are designated with lower case letters. (*B*) VH31, an NP^b-like pseudogene from BALB/c (87). Asterisks denote stop codons in its sequence. (*C*) VH283, a germline gene from the 7183 family (88). (*D*) 6G6, a rearranged V_HS107 family gene cloned from a CBA hybridoma (89), and pH3-6a, a cDNA clone from C57Bl/6 splenic mRNA (90); the sequence is from the Vgam3 family. In (*A*), the sequence of 1A11 has been corrected compared with Shlomchik et al. (20). These sequence data have been submitted to the EMBL/GenBank Data Libraries.

assigned to sets with multiple members (i.e., "singlets"). Of course, singlets would be found in a small sample of cells from a mouse made up, concurrently, of clones of different sizes. Mouse 5, with three clones (of size 3, 2, and 2) illustrates this. Thus, a singlet could represent an expanded clone of fewer cells than clones from which we have found multiple examples. Alternatively, they could represent cells without siblings.

Ig V_H and V_κ Usage. Ig V gene usage can be interpreted in the context of the inherited V gene repertoire of inbred mice. At present, 13 V_H and 30 V_κ groups (based on >80% nucleotide sequences homology) have been identified (33–39). It is estimated by Southern hybridization that there are ~125 V_H and ~150–250 V_κ genes (33, 40). Our panel of 31 antibodies (combined with the nine examples described by others [36, 41, 42]) shows that anti-DNA antibodies are encoded by at least five different V_H families and 13 different V_κ groups (Table I).

In spite of the V gene diversity among DNA antibodies, certain V gene preferences emerge in this extensive survey. A single J558 V_H gene is probably used in three separate clones. D20, clone A, and clone E are 96% homologous, and closely homologous to a V_H gene, 133.16, described previously (43) (Fig. 1 *A*). The restriction fragment length of the rearranged allele in the three clones is the same (adjusting for different J_H usage), also consistent with their utilizing the same V_H gene (data not shown). DNA blotting data with oligonucleotides overlapping the mutations at sites 160 and 173 in 3H9 confirms this (Mascelli, M. and M. Weigert, manuscript in preparation). This V_H gene is preferentially selected; assuming 100 germline V_H genes, the likelihood of choosing the same gene three times in 15 samples by chance is 0.03. Another possible preference is for $V_\kappa 8$ in that different members of this group are found in 3 of the 15 independently derived sets. This frequency would indicate a significant bias ($p = 0.05$) if the $V_\kappa 8$ group constituted not >5.7% of the V_κ repertoire. However, $V_\kappa 8$ is thought to comprise at least 10% of the V_κ gene repertoire (40), therefore this $V_\kappa 8$ incidence does not represent a significant bias.

The V_κ of clone D is just 77% homologous to $V_\kappa 9$ (44), its closest relative, and therefore does not meet criteria for assignment to a known V_κ group. We therefore assign it a new V_κ group, 31. Finding a new V_κ group in this survey of antibodies to a novel antigen does not necessarily mean that this group is uniquely associated with the DNA antibodies of the autoimmune mouse, as statistical models predicted that six V_κ groups were as yet unidentified (39).

J_H, D_H, J_κ Usage and Junctional Diversity. No restriction to a particular J_H, J_κ, or D_H segment was seen: all J_H, J_κ gene segments, as well as representatives from each D_H family, were found (Table I). This variety of gene segment usage leads to considerable phenotypic variation of the V_H CDR3 of these antibodies, both in amino acid composition and length. Two unusual features of anti-DNA V_H CDR3s, relative to other antibodies, were noted. One is similar to the observation of Eilat et al. (42) that the anti-DNA antibodies A52 and D42 read their D_H segments in a frame different from other antibodies. As noted by Kaartinen and Mäkelä (45), most antibodies read Ds in the same frame. Here, 5 of the 13 independent clones (S106, D20, DP1, clone E, and clone F) show a D reading frame shifted one nucleotide backward or forward from the preferred reading frame. One interesting consequence of this shift is that in three cases (clone E, clone F, and S106), these reading frames yield arginine codons.

A second unusual feature of anti-DNA or V_H CDR3s is that fusion of two D segments may have occurred during V_H assembly. D-D fusion has been previously invoked to explain sequences of CDR3 of other antibodies (18, 46–48). As illustated in Fig. 1, *C* and *D*, for antibodies 6O and DP12, one of the Ds participating in the fusion is inverted relative to the other segments. For 6N (Fig. 1 *A*) the CDR3 segment is formed by the fusion of two inverted D gene segments. The 5′ D segment matches the noncoding strand of the SP2 core sequence in seven of eight nucleotides, and the 3′ region matches the noncoding strand of FL16-1 in seven of eight nucleotides. The 5′ CDR3 region of 6O is a perfect match of six nucleotides with an SP2 gene segment and the 3′ region matches 8 of 10 nucleotides of the noncoding strand of a different D segment. Likewise, the 5′ CDR3 region of DP12 is a perfect match of seven nucleotides with an SP2 gene, and at the 3′ end, six of seven nucleotides match the noncoding strand of SP2 -5, -7, or -8. In two of these three examples, inverted Ds encode arginine residues.

Somatic Mutation. As shown in Figs. 1 and 2, there are numerous intraclonal differences, indicative of somatic mutation during clonal expansion. These intraclonal comparisons provide a minimum estimate, because mutations shared by all members of a clone will not be recognized. However, as discussed above for clones A, E, and D20, the likely germline donor V_H sequence is known, and the exact frequency of mutation can be determined. The comparison of the V_H genes of clone A to the germline V_H (Fig. 1 *A*) reveals a shared mutation not apparent from intraclonal comparison. We surmise that this mutation represents an early event in the expansion of this clone. D20, a singlet, is identical to the germline V sequence.

Specificity Analysis. Anti-DNA detected by screening hybridoma supernatants for binding to denatured calf thymus DNA were further analyzed by both solid and solution phase DNA binding assays. These assays confirmed the initial screen in that all examples bound denatured ssDNA (20, 21, Table II, and data not shown).

Most members of clones (with certain exceptions discussed below) had indistinguishable binding activities for ssDNA by either assay, for instance, clones C, E, and F (Table II, and data not shown). Interclonal comparisons, on the other hand, showed pronounced differences. This can readily be seen in Table II for ssDNA binding of clones D and F. Since these antibodies are the same H chain isotype, these differences are attributable to V gene sequence. Such interclonal differences are consistent with the high degree of V region diversity between clones.

Interclonal differences in dsDNA binding were also apparent, including several examples for which no dsDNA binding was detectable (Table II). Nevertheless, one or more members of each clone bound dsDNA. Thus, at the levels of hybridomas, this characteristic serologic feature of autoimmunity is also seen. Among the singlets, a clear distinction is seen between the IgM and IgG antibodies. Whereas all IgG singlets bound dsDNA, the IgM singlets only bound ssDNA. In this respect, these IgM antibodies are similar to the anti-DNA seen in normal individuals and may result from a different activation mechanism than the IgG anti-DNA typical of autoimmune disease.

Intraclonal differences are seen for both ds- and ssDNA binding. These differences can only result from somatic mutation, therefore, the structural basis for differences in specificity can be attributed to limited amino acid substitutions. These substitutions often include mutations to arginine or asparagine, amino acids known

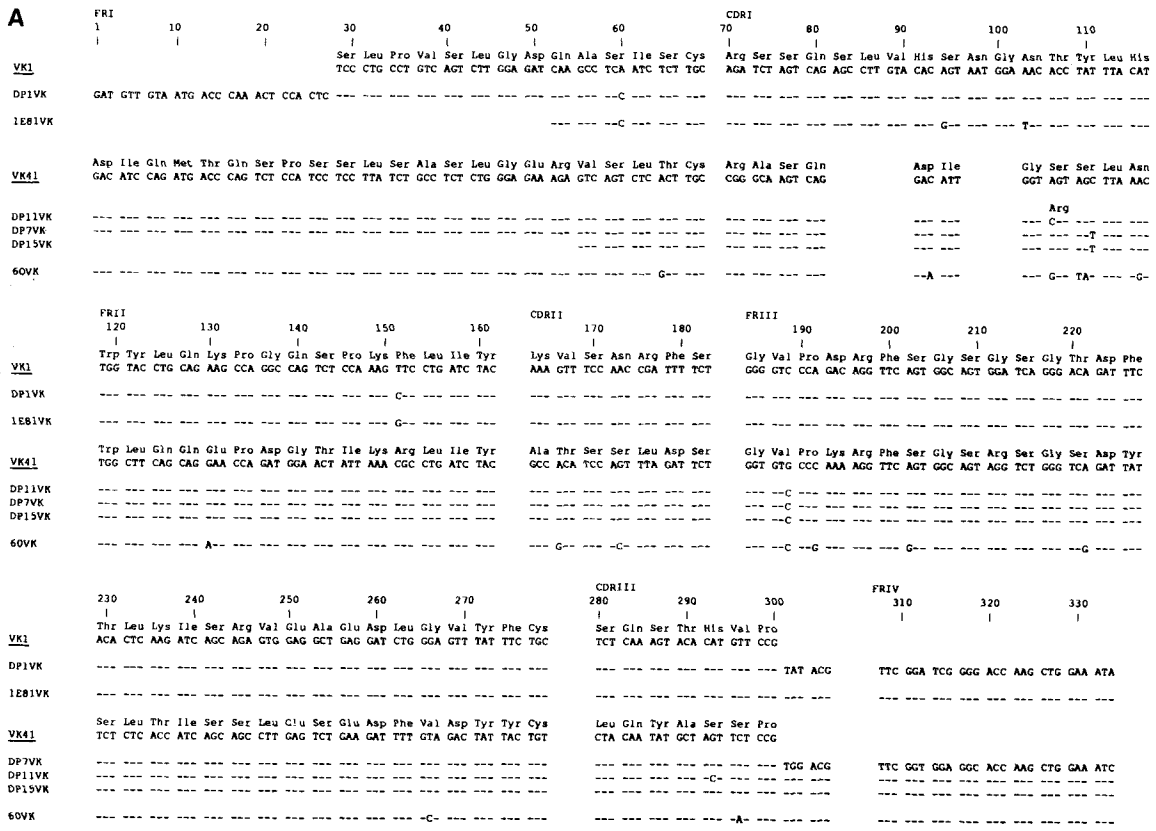


FIGURE 2. Legend on page 280.

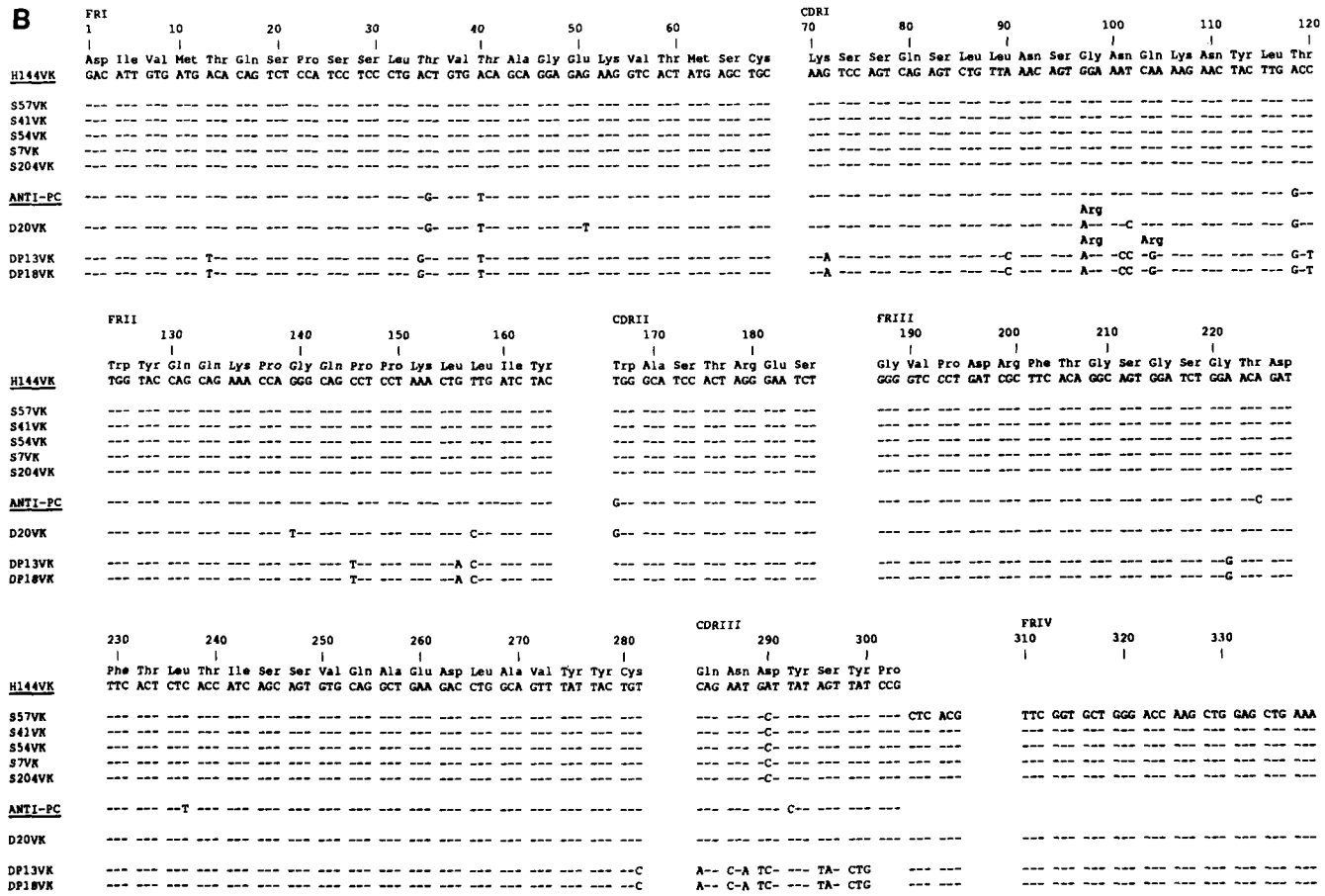
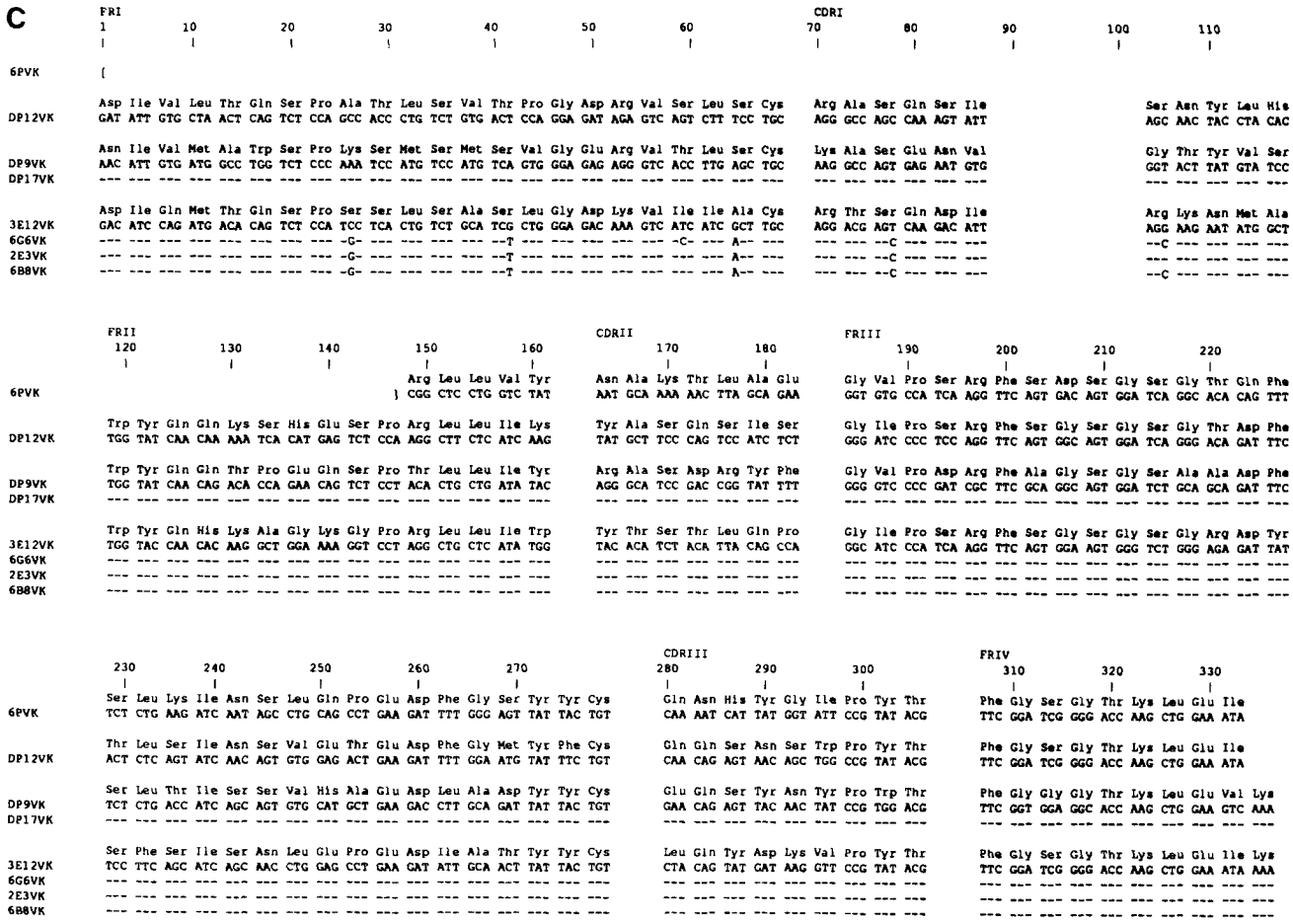


FIGURE 2. Continued.

C



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D

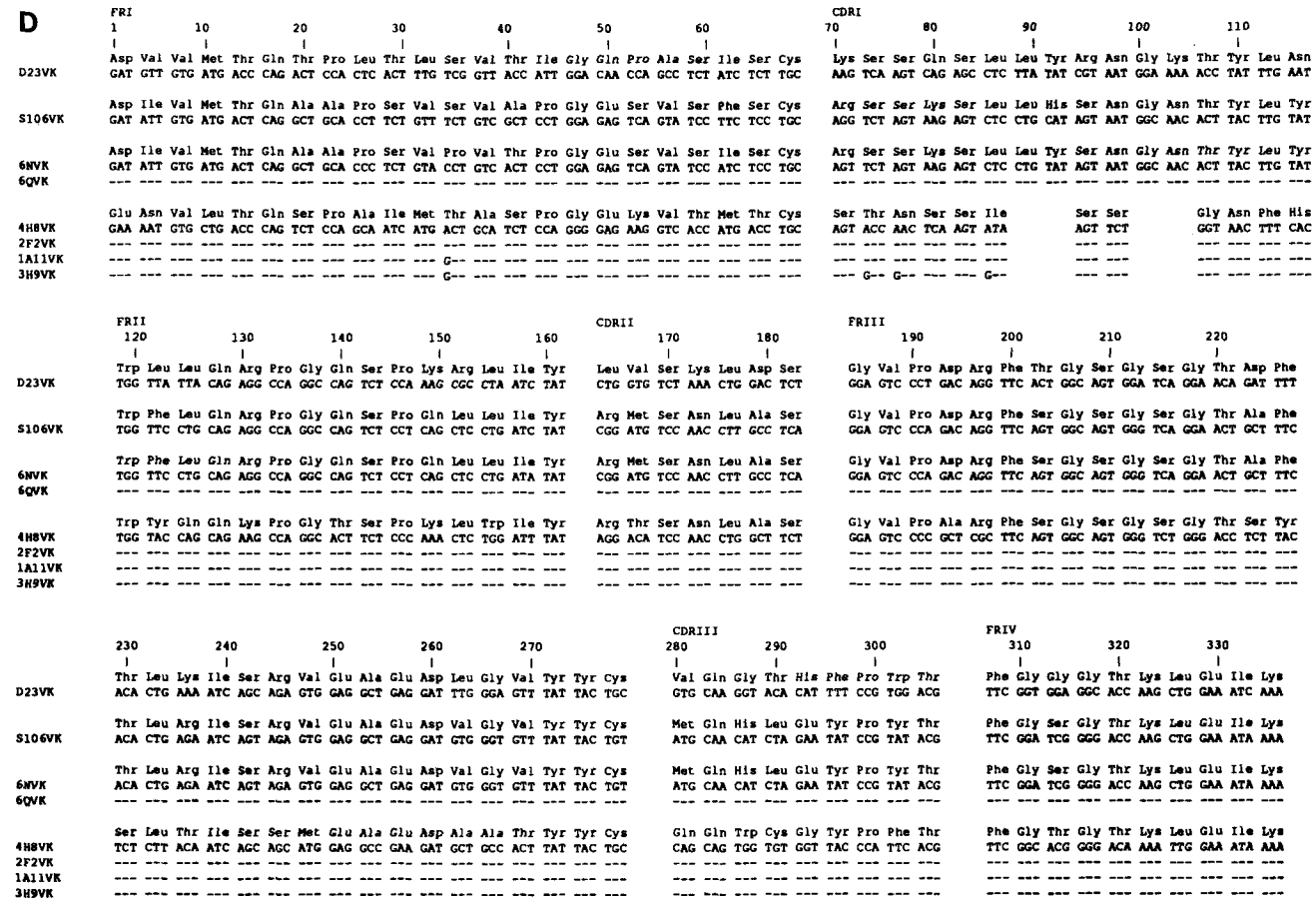

FIGURE 2. Continued.

to play a central role in DNA-protein interactions (49, 50). For example, 3H9, a member of clone A, has acquired an additional arginine in CDR2 of its H chain. This arginine could account for the unique ability of this member of clone A to bind dsDNA, as well as to bind ssDNA with higher affinity than the other members of clone A. DP7 and DP11 (clone E) both bind dsDNA but differ significantly in affinity. Again, the somatic mutations that must determine this difference include a serine to arginine replacement in CDR1 of the V_κ of DP11. However, this mutation did not lead to a significant difference in affinity for ssDNA. Thus, even though arginine has the potential for binding to epitopes unique to dsDNA, as well as epitopes shared by both ds- and ssDNA (such as phosphodiester linkages), the arginine of DP11 may be constrained to interact just with dsDNA moieties.

Discussion

MRL/*lpr* mice develop a progressive autoimmune syndrome serologically and pathologically similar to human SLE (2, 10, 11, 14, 51). To examine the B cells that secrete these pathologic autoantibodies, we have constructed hybridomas from unmanipulated splenic lymphocytes of unimmunized older MRL/*lpr* mice. In these mice, the frequency of such spontaneous hybridomas that secrete self-specific antibodies (e.g., anti-dsDNA [this work] and anti-Ig [52]) is much higher than the frequency among preimmune LPS-stimulated B cells of several normal mouse strains (53–55) or unmanipulated B cells of normal C57Bl/6 × DBA/2 F_1 mice (56). We have determined the specificity and V region sequences of monoclonal anti-DNA autoantibodies from five different MRL/*lpr* mice. These data allow us to draw several important conclusions about the mechanism of humoral autoimmunity in MRL/*lpr*, the genetic basis of anti-DNA specificity, and the possible structural basis for idiotypes.

Pathologic Anti-DNA Are the Result of Oligoclonal B Cell Expansion

Since the anti-DNA repertoire in a mouse is large (54, 55, 57), a polyclonal activation model would predict a multiclonal response. However, at least half of the hybrids from each of the mice were members of expanded clones. Therefore, the model of autoimmunity that accounts for anti-DNA production via polyclonal activation is inconsistent with our data, and this model does not apply to the MRL/*lpr* syndrome.

Clonal Expansion Is Specific and Receptor Driven

The oligoclonal composition of the anti-DNA from each mouse establishes that the activation and propagation of DNA-specific B cells in MRL/*lpr* mice is selective. Although the selective expansion of certain B cells could be independent of the antigen receptors, our data indicate that for anti-DNA, selection is dependent on receptor specificity. This conclusion comes from features of the genes encoding the receptors

FIGURE 2. Nucleotide sequences of L chain (V_κ) regions of anti-DNA antibodies. The sequences are organized as in Fig. 1. Sequences from the same V_κ group as defined by Potter et al. (39) are together. In some cases a prototype and its translation are given; where no prototype is given, the topmost sequence of each homology group is translated. Prototypes are: (*A*) $V_\kappa 1$, a BALB/c germline gene from this group (91), and $V_\kappa 41$, a BALB/c germline gene from the $V_\kappa 9$ group (92); and (*B*) H144V_κ, an mRNA sequence from an influenza hemagglutinin-specific IgM hybridoma expressing the consensus sequence of a $V_\kappa 8$ group gene (Clarke and Weigert, manuscript submitted for publication), and anti-PC, a consensus from a set of highly homologous sequences from PC-specific antibodies thought to all express a single (different) $V_\kappa 8$ group gene.

TABLE II

Solution Phase DNA Binding of mAbs

Mouse	Cell	Clone	50% Maximal binding ssDNA	50% Maximal binding dsDNA	ss/dsDNA
			μg/ml		
1	1A11	A	0.55 ± 0.02	>1.1	-
1	4H8	A	0.57 ± 0.04	>4.0	-
1	3H9*	A	0.12 ± 0.01	0.32 ± 0.04	0.4
3	S106		2.2 ± 0.20	1.55 ± 0.15	1.4
3	D20		0.65 ± 0.07	0.43 ± 0.04	1.4
3	S7	C	2.5 ± 0.60	2.3 ± 0.80	1.1
3	S41‡	C	ND	ND	
3	S54‡	C	ND	ND	
3	S57	C	3.8 ± 1.0	3.1 ± 0.20	1.2
4	1E81		0.03 ± 0.00	0.54 ± 0.05	0.06
4	3E12	D	0.62 ± 0.04	0.34 ± 0.02	1.8
4	6G6	D	0.60 ± 0.04	0.33 ± 0.06	1.8
4	68B	D	0.72 ± 0.05	0.52 ± 0.14	1.4
4	3G9	D	0.76 ± 0.06	0.42 ± 0.06	1.8
4	2E3	D	0.71 ± 0.06	0.45 ± 0.07	1.6
5	DP7§	E	0.38 ± 0.03	0.58 ± 0.18	0.7
5	DP11‖	E	0.34 ± 0.05	0.15 ± 0.02	2.3
5	DP9	F	0.13 ± 0.01	0.10 ± 0.01	1.3
5	DP17	F	0.12 ± 0.01	0.09 ± 0.00	1.5
5	DP13	G	0.07 ± 0.02	>2.2	-
	H241¶		1.0 ± 0.20	0.90 ± 0.10	1.1
	C11**		0.016 ± 0.02	>6.0	-

Native and denatured DNA specificity was assayed according to the procedure outlined in Materials and Methods. Triplicate determinations were performed for each concentration of antibody. Antibody concentrations at half-maximal binding are determined by extrapolation from the linear portions of the binding curves. The confidence interval for a value of the independent variable (antibody concentration), based on a single value of the dependent variable, was determined by simple linear regression using the IMSL subroutine RINPF, as discussed by Graybill (86). The IgM antibodies (DP1, DP12, and D23) did not give detectable binding in this assay for either form of DNA. Antibodies 1A11, 4H8, and DP13 showed no significant binding to ssDNA. (>) The highest antibody concentrations assayed for which no binding was detected.

* Values for ssDNA and dsDNA are significantly different from other clone members.

‡ The antibody concentrations of supernatants of S41 + S54 were too low to generate binding curves.

§ Value for dsDNA is significantly different from DP11.

‖ DP11 is unusual because maximal binding to ssDNA was lower than the other antibodies. The epitope on ssDNA recognized by this antibody may be limiting. As we only wish to demonstrate relative binding to ssDNA without regard to the precise epitope, we have normalized the maximum A405 reading of DP11 to that achieved by C11, our standard for maximal ssDNA binding.

¶ H241 is anti-ssDNA and anti-dsDNA (27).

** C11 is anti-ssDNA (24).

TABLE III

Anti-DNA V_H CDR3 Amino Acid Sequences

Name	V_H	CDR3		J_H	C_H
60	Vgam3	s**R**yy**R**y**R**	mfdy	4	IgG2a
6N (2)	J558	y**RR**llp	fay	2	IgG2a
DP9 (2)	J558	ngl**RRR**	awfay	3	IgG1
MRL22*	J558	**R**ly**R**	yyamdy	2	IgM
BXW14*	J558	**R**gttv**R**d	dy	2	IgM
D42*	S107	ggl**RR**g**R**s	fdv	1	Ig2a
A52*	J558	g**R**l**RR**gg	yfdy	2	IgG2b
2E3 (5)	7183	**R**dyshwf	fdy	1	IgG1
1A11 (4)	J558	a**R**skysyv	mdy	4	IgG3
S57 (5)	J558	**R**gtlgkg	yfdy	2	IgG3
DP7 (2)	J558	n**R**	wfay	3	IgG3
S106	7183	ggt**R**vg	awfay	3	IgG3
D20	J558	sgl**R**glglp	fay	1	IgG2a
1E81	J558	**R**shy	awfay	3	IgG2a
BXW7*	J558	**R**ayygssph	wyfdv	1	IgM
H130*	J558	s**R**aysny	yamdy	4	IgM
D23	J558	ednyg	fay	3	IgM
DP1	S107	lw	ay	3	IgM
DP12	J558	lygai	dy	2	IgM
MRL10*	J558	lvgg	gfay	3	IgM
MRL4*	10	daanwsa	wfay	3	IgG2a

The antibodies in this survey and published anti-DNA sequences are included. Each clone is represented by only one cell line. Numbers in parentheses after the name indicate the total number of hybridomas from that clone; singlets are not given numbers. In the CDR3 column are given the single letter amino acid sequences of V_HCDR3s (as defined by Kabat et al. [44]). Arginine residues (**R**) are capitalized and bolded. The portion of the CDR3 sequence encoded by the J_H region, as inferred by nucleotide sequence homology, is listed to the right, separated from the remainder of CDR3 by spaces. The V_H family, J_H gene, and C_H gene used are given in separate columns; assignments are the same as in Table I. Sequences are listed in order of descending arginine content. The five sequences at the bottom do not contain arginines. MRL22, BXW14, BXW7, MRL10, and MRL4 are taken from Kofler et al. (36); D42 and A52 are from Eilat et al. (42) and H130 is from Trepicchio et al. (41).

* Sequences from other sources.

themselves: the nonrandom distribution of somatic mutations found in anti-DNA clones, the nature of the replacement (R) mutations found in several of the clones and their effect on the specificity of the antibodies, and unusual sequences of the V_HCDR3 regions.

Nonrandom Patterns of Somatic Mutation. V regions from antigen-activated B cells have characteristic, nonrandom patterns of somatic mutations (28, 58). These patterns must result from positive selection of cells with R mutations in CDRs, presumably because some of these mutations improve affinity for antigen (32, 59, 60). This process often leads to a population of cells with a higher percentage of R mutations in CDRs than can be explained by chance alone. We find that autoantibodies have similar skewed patterns of mutations: nonrandom mutation is present among multiple clones of RFs derived from autoimmune mice, as we previously reported, and in clone A (19, 20). A nonrandom pattern ($p = 0.03$, analysis as described in

Shlomchik et al. [20]) is also found in Clone E. These improbable distributions can be accounted for by selection, but not by receptor-independent stimulation. The other clones shown are uninformative in this regard because we cannot identify enough mutations to permit a meaningful analysis of the distribution.

Specificity for dsDNA. In humans and mice, the preimmune repertoire includes mainly ssDNA-specific B cells and few dsDNA-specific B cells (7, 54, 55). We selected the hybridomas on the basis of ssDNA binding alone. In the absence of preferential in vivo expansion of dsDNA-specific cells (i.e., selection), we would have expected mainly anti-DNA specific for ssDNA only. Instead, most of the antibodies that we recovered recognized both ss- and ds-DNA. As others have noted (61–63), this result (frequent recovery of what is a rare specificity in the preimmune repertoire) can readily be explained by selection for anti-dsDNA. Nonspecific activation models could also explain this observation if a subset of B cells enriched for certain self-specificities (such as dsDNA) were preferentially (but nonspecifically) activated during autoimmunity. However, such a B cell subset model cannot explain our results, since in two separate cases (clones A and E), we have found descendents of a single B cell precursor that differ dramatically in their ability to bind dsDNA. Thus, ds- and ssDNA-specific autoreactive cells can be derived from the same precursor B cell.

Somatic Mutations Can Cause dsDNA Specificity and Are Selected During Clonal Expansion. The differences in specificity among members of clones A and E must be due to somatic R mutations (Figs. 1 and 2). Certain features of these mutations argue for positive selection of mutant receptors. First, somatic mutations in 3H9 and DP11 lead to better dsDNA binding. Thus, in at least two cases clones have evolved toward higher affinity for dsDNA. Second, the small number of such mutations that nonetheless caused improvement in affinity for dsDNA suggests that selection for these mutants took place in vivo. We infer this because the number of mutations that can improve affinity is small relative to the number of possible silent mutations, R mutations in conserved sites that destroy antibody function (~35%, [20]) and the fraction of R mutations in CDRs that adversely affect binding. Thus, in an unselected population of cells, each with a few random mutations, as would result from receptor nonspecific activation, antibodies with enhanced binding would be rare. However, selection would enrich for such mutants and could explain the observed pattern of mutations. Third, the nature of individual mutations in these and other clones appears to reflect a predictable consequence of selection by DNA: there is a high frequency of mutations leading to arginine and asparagine residues, amino acids known to play a role in binding to DNA (49, 50). In clone E, of seven total R mutations, there are four independent changes to arginine and one to asparagine. Of the three unique replacement mutations that occurred in 3H9, one (in CDR2) was a change to arginine. Strikingly, in two cases, independent mutations occurred in the same codon of the clone E V_H; two different base changes generated serine to arginine mutations at codon 76 and separate base changes converted serine to arginine in one case and to asparagine in the second case at codon 31 (Fig. 1).

Selection for Arginines Is a Common Feature of Anti-DNA. In addition to finding arginines generated by somatic mutations, we identified arginines at sites where this residue has never or rarely been found in large collections of sequences of other V genes from the same gene families (44, 64, 65). These unprecedented arginines are likely to be the result of somatic mutations. These are the contiguous arginines in V_HCDR2 of clone D, position 64 of V_HCDR2 of clone B, position 27F of the V_κ

CDR1 of D20, DP13, and DP18, and position 29 of the V_κCDR1 of DP13 and DP18. It is of interest that all occur at sites in the combining site.

The V_HCDR3s of these antibodies also contain many arginines: out of 339 CDR3 residues, 46 were arginine (among 37 sequences, including the nine sequences from the literature [36, 41, 42]). All but five sequences had one arginine and five had two, four had three, and one had four arginine residues in CDR3 alone. The large pool of known V_HCDR3 sequences (as compiled by Kabat et al. [44]) allows a reasonable estimate of the random frequency of arginines in this region, thus making it possible to test whether the arginine content in this region of anti-DNA is significantly higher than expected. There were 105 arginines in 3,066 CDR3 residues in the Kabat et al. compilation. A χ^2 analysis of the frequency of arginines in the Kabat et al. data and in these anti-DNAs gives a χ^2 of 76 (1 df, $p < 5 \times 10^{-7}$).

Unusual mechanisms appear to explain the generation of arginine codons during somatic V region assembly, including out-of-frame D joining and D-D fusion. In addition, some of the arginine codons arise from nontemplated bases (N regions, [31]). Unlike the somatic mutations to arginine that occur during the antigen driven phase of B cell differentiation, the CDR3 arginines were formed at the earlier pre-B cell stage of differentiation, when V assembly occurs. Therefore, the process of selection that resulted in enrichment of arginines in V_HCDR3 probably began at or near the beginning of clonal expansion and continued during the phase of accumulation of somatic mutations. Since these V_HCDR3 arginines and those formed at later stages by somatic mutation can both be selected, the loss of regulation against self-specificity in MRL/*lpr* autoimmunity is ongoing, and probably encompasses multiple stages of B cell activation.

The Meaning of Single Isolates

One in four of our hybrids are "singlets," which cannot be assigned to sets with multiple members. Since clonal size cannot be estimated from singlets, they could represent very small clones. If singlets do indeed represent many very small clones, these in turn could result from either polyclonal activation or recent antigen activation. Polyclonal activation could account for the fact that three of the singlets in this study lack the features of selection described above: they are IgM, lack CDR3 arginines, and do not bind dsDNA. On the other hand, the singlets that do have these features may have come from expanded clones from which we isolated just one example.

If in vivo polyclonal activation does lead to autoantibody production, as claimed by Klinman and colleagues (66, 67), our data show that its role is limited in two regards. First, it would have to exert its effect only early in the process of self-specific B cell activation, since the current studies clearly establish that oligoclonal expansion is the proximate event to the development of pathogenic-type IgG autoantibodies. Therefore, any changes in B cell differentiation state due to polyclonal activation would only serve to facilitate this process, perhaps by lowering the threshold for antigen activation or allowing B cells to bypass the usual control(s) over self-recognition. Second, even if all of the single isolates did represent polyclonally activated B cells, their contribution to the pool of DNA-specific activated B cells in older MRL/*lpr* mice is minor, since they comprise only about one-quarter of all our anti-DNAs and of our RFs (19).

Genetic Control of Anti-DNA Specificity

We have identified several strategies that contribute to DNA binding. Germline V genes must in part encode the ability to bind DNA; although many different V genes are used in anti-DNA, we do not expect that all or even most V genes will be capable of encoding DNA specificity. This is supported by the fact that the representation of V genes in our anti-DNA sample is not entirely random, since at least one V gene is found more frequently among anti-DNA than expected. The frequency of this V_H suggests it plays a role in DNA binding. However, as some of the antibodies expressing this V_H bind only ssDNA (e.g., 1A11), while others bind dsDNA, other elements must also contribute. The nature of the V_HCDR3 sequences, as discussed, strongly implies that this region also plays a role in binding in at least some antibodies. Somatic mutations are another factor, as shown by the two cases of mutations in the V_H improving and/or creating dsDNA specificity (clones A and E). Indeed, these two observations provide the first direct evidence of in vivo, stepwise improvements of binding via somatic mutation.

Improvements of DNA binding are correlated with arginine substitutions in at least four sites. Further, although most of the anti-DNAs have an arginine-containing CDR3, the locations of these CDR3 arginine residues are different in each case. Evidently, arginine residues at multiple sites can affect DNA binding. In this regard, DNA may be unlike the hapten nitrophenyl; increased affinity for nitrophenyl is associated with mutation at one recurrent site (60). The variety of sites at which unusual or mutant arginines appear suggests to us that antibody binding of dsDNA may be built up additively in a way reminiscent of antibodies to oligosaccharides (68, 69). For example, it may be that an arginine anywhere in the combining site can contribute to binding. This seems plausible given the polymeric nature of the DNA antigen and the fact that arginine can interact with at least three different groups on DNA (phosphodiester backbone, base-paired guanine, and unpaired and base-paired cytidine) (49, 50), which in turn could be expressed in a variety of conformations (i.e., epitopes).

Implications for Idiotypes

Several groups have produced antiidiotypic antibodies that recognize a majority of anti-DNA in sera of autoimmune mice and humans (70–76). These studies suggested that since a majority of anti-DNA shared a serologically defined similar structure, anti-DNA would be encoded by a restricted set of V genes. However, in three separate cases (42, 77, 78), idiotypes did not correlate with V_H family when idiotype-positive molecules were sequenced or typed for V_H family. Our study extends these findings by showing that anti-DNA are not encoded by a restricted set of V genes; even the most frequently expressed V_H accounts for only 25% of known anti-DNA sequences. It thus seems unlikely that shared idiotypes can be explained by common expression of a V gene. Similarly, it is unlikely that antiidiotypes that recognize the majority of anti-DNA could be "internal images" of DNA (79), because the diverse binding specificities of anti-DNA (57) indicate that there must be many distinct internal images. Thus, the structural explanation for idiotypes lies elsewhere.

We suggest that high arginine content could explain some recurrent antiidiotype systems. For example, arginine-rich CDR3s (or other arginine-rich CDRs) are a feature shared by many anti-DNAs, and these could be recognized by some antiidi-

otypic antibodies. This hypothesis can now be tested by correlating these idiotypes to sequences. In this regard, Eilat et al. (42) recently showed that the V_H sequences of two anti-DNAs that share an idiotype differ at V_H and V_K, but have similar, arginine-containing CDR3s.

This comprehensive survey allows us to draw several conclusions about the etiology and genetic basis of anti-DNA in MRL/*lpr* mice: most or all of the anti-DNA in a given animal are the product of one or a few clonally expanded B cells whose V genes have somatic mutations. The stimulus for this clonal expansion is mediated via the antigen receptor. Combined with the similar results with MRL/*lpr*-derived RFs, this study shows that antigen-driven clonal expansion is a common feature of humoral autoimmunity in MRL/*lpr* mice.

B cells in MRL/*lpr* autoimmunity closely resemble B cells in normal contexts participating in T cell–dependent secondary immune responses (28, 58, 80). It seems likely from this similarity that autoimmune B cell clonal expansion is also dependent on Th cells. This suggests that the primary defect may not be in the B cell per se, but instead, in regulation of the B cell by T cells or T cell–derived factors (e.g., excessive T cell help or absence of T cell suppression). In particular, a search for an activity that facilitates antigen-driven B cell activation should be fruitful.

The antigen stimulus in this case is likely to be DNA, because in two cases affinity for DNA has improved during clonal expansion and evolution. Indeed, in most of the sequences, there is preferential utilization of the amino acid residues expected to play a role in DNA binding. Both these features are unexpected unless DNA itself is the B cell antigen.

Some V genes are found more frequently than expected among antigen-driven anti-DNA, but no V genes or V gene pairs are expressed frequently enough to account for idiotypes expressed on the majority of anti-DNA. This results in a diversity of anti-DNAs sufficient for each individual mouse to have a unique set of anti-DNA clones at any given time. Since the human immune system contains many more cells, such diversity will be recapitulated in a single person. Furthermore, even members of a single clone will differ due to accumulated somatic mutations. These two sources of diversity should make it unlikely that approaches to treatment using antiidiotypic antibodies as specific reagents against pathogenic anti-DNA will eliminate a significant proportion of these autoantibodies in any single individual. Two early attempts at this therapy were unsuccessful in achieving long-term suppression of anti-DNA (81, 82). Indeed, even in a monoclonal B cell proliferative disease (follicular lymphoma), the somatic mutation process alone generated sufficient diversity to prevent elimination by antiidiotypic antibody therapy (83). However, we do note that anti-DNA Vs are arginine rich, particularly in V_H CDR3; we propose that arginines could in part be the ligands for antiidiotypic sera. This suggests that a more promising therapy might use smaller aromatic and charged molecules aimed at the building blocks of DNA specificity (e.g., arginines in certain contexts), as has been explored by Ben-Chetrit et al. (84) and Shojania and Orr (85). Further genetic, functional, and crystallographic analysis of these and similar antibodies and in vitro generated mutants of them will lead to a better understanding of the rules governing antibodies binding to DNA and ultimately the informed design of compounds that might specifically inhibit this interaction.

Summary

The proximate cause of autoantibodies characteristic of systemic autoimmune diseases has been controversial. One hypothesis is that autoantibodies are the result of polyclonal nonspecific B cell activation. Alternatively, autoantibodies could be the result of antigen-driven B cell activation, as observed in secondary immune responses. We have approached this question by studying monoclonal anti-DNA autoantibodies derived from unmanipulated spleen cells of the autoimmune MRL/*lpr* mouse strain. This analysis shows that anti-DNAs, like rheumatoid factors (19), are the result of specific antigen-driven stimulation. In addition, correlation of sequences with fine specificity shows that: (*a*) somatic mutations can cause specificity for dsDNA and that such mutations are selected for; (*b*) arginine residues play an important role in determining specificity; and (*c*) antiidiotypes that recognize the majority of anti-DNA are probably not specific for any one family of V regions.

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