Both IgM and IgG Anti-DNA Antibodies Are the Products of Clonally Selective B Cell Stimulation in (NZB × NZW)F1 Mice

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Summary

Disease activity in systemic lupus erythematosus is closely associated with the appearance of immunoglobulin (Ig)G antibody to native DNA in both humans and mice. Like normal antibody responses, the anti-DNA autoantibody first appears as IgM and then switches to IgG. Structural studies of IgG anti-DNA suggest that these antibodies are the products of clonally selected, specifically stimulated B cells. The origins of the IgM anti-DNA have been less clear. To determine whether the earlier appearing IgM anti-DNA antibody in autoimmune mice also derives from clonally selected, specifically stimulated B cells or B cells activated by nonselective, polyclonal stimuli, we have analyzed the molecular and serological characteristics of a large number of monoclonal IgM anti-DNA antibodies from autoimmune (NZB × NZW)F1 mice. We have also analyzed IgM and IgG anti-DNA hybridomas obtained from the same individual mice to determine how the later-appearing IgG autoantibody may be related to the earlier-appearing IgM autoantibody within an individual mouse. The results demonstrate that: (a) IgM anti-DNA, like IgG, has the characteristics of a specifically stimulated antibody; (b) IgM and IgG anti-DNA antibodies have similar variable region structures and within individual mice may be produced by B cells derived from the same clonal precursors; (c) recurrent germline and somatically derived VH and VL structures may influence the specificity of anti-DNA monoclonal antibody for denatured vs. native DNA; and (d) the results provide a structural explanation for the selective development of IgG antibody to native DNA as autoimmunity to DNA progresses in (NZB × NZW)F1 mice.

Antibody to DNA is a manifestation of the autoimmune disease SLE and plays a demonstrable role in disease pathogenesis in both humans (1) and (NZB × NZW)F1 mice (2, 3). Because of similarities to humans in the development of disease, (NZB × NZW)F1 mice have become useful experimental models for studying the cellular and molecular basis for anti-DNA autoantibody production (3). In particular, the availability of hybridoma-derived, anti-DNA mAbs from autoimmune mice has provided the opportunity to analyze the structural basis for antibody specificity to DNA (4). How anti-DNA antibody originates both in human and mouse SLE remains obscure, however.

Previous experiments from our laboratories have concentrated on the analysis of interclonal and intraclonal diversity of spontaneous anti-DNA antibodies within an individual autoimmune (NZB × NZW)F1 mouse (5–7). The results from those analyses demonstrated that the IgG anti-DNA antibody within an individual autoimmune mouse had all the characteristics attributable to clonally selected, secondary immune antibody to specific antigen: IgG isotype, oligoclonality, and V region somatic mutations among clonally related antibodies. The results also suggested that the antigen most likely to have stimulated such an antibody was DNA. In this regard, anti-DNA in (NZB × NZW)F1 mice appears to be similar to anti-DNA in MRL lpr/lpr mice (8, 9). However, the results from at least one study on the clonal heterogeneity of anti-DNA antibody within individual autoimmune (NZB × NZW)F1 mice led to a different conclusion (10). Although Vh, and Vδ gene representation among the hybridomas in the previous study was not consistent with a polyclonal population, the hybridomas were nonetheless clonally diverse. Anti-DNA antibody in autoimmune mice undergoes a spontaneous isotype shift from IgM to IgG (11–13). Analyses of B cell activity in (NZB × NZW)F1

1 Abbreviations used in this paper: VH, immunoglobulin heavy chain variable region; Vδ, heavy chain variable region gene; VL, immunoglobulin light chain variable region; Vδ, light chain variable region gene.
mice (14) have led to the hypothesis that at least the initial, IgM stage of anti-DNA autoantibody production is due to polyclonal activation of B cells (15) and that subsequent clonal selection of IgG anti-DNA-producing B cells occurs subsequent to this event. However, there have been no direct studies to determine whether IgM anti-DNA autoantibodies have the characteristics of antibody produced by a monoselected, polyclonal population of B cells. Likewise, there have been no studies to directly determine the clonal relationship between the IgM and IgG anti-DNA autoantibodies within individual (NZB × NZW)F1 mice.

The present experiments were proposed to accomplish three major goals. (a) Since there has been some question as to whether the autoimmune anti-DNA antibody in (NZB × NZW)F1 mice is generally oligoclonal and characteristic of an antigen-stimulated, secondary immune response, we extended our analysis of the clonal diversity of IgG anti-DNA antibodies to include seven additional (NZB × NZW)F1 mice. (b) To determine whether the earlier-appearing IgM anti-DNA antibody is also clonally selected and expresses V region structures that are similar to the later-appearing IgG anti-DNA antibodies in autoimmune (NZB × NZW)F1 mice, a large number of IgM anti-DNA hybridomas from four different mice were analyzed. (c) To determine whether the IgG anti-DNA antibody within an individual autoimmune mouse is clonally related to the earlier-appearing IgM anti-DNA antibody within the same mouse, IgM and IgG hybridomas from three individual mice were analyzed. The results confirmed our previous results that spontaneous IgG anti-DNA antibodies within the same mouse, IgM and IgG hybridomas from four different mice were analyzed. The results confirmed our previous results that spontaneous IgG anti-DNA antibodies in (NZB × NZW)F1 mice are generally oligoclonal in origin and have V region structural characteristics consistent with an antigen-selected derivation. The results also demonstrated preferential expression of particular Vh and Vk genes among IgM anti-DNA hybridomas, suggesting that IgM anti-DNA is also produced by selective B cell stimulation. Finally, the results demonstrated that in general IgM and IgG anti-DNA autoantibody-producing B cells have structurally similar Ig V regions and that within an individual mouse at least some of the IgG anti-DNA antibody-producing B cells are derived from the same clonal precursors as the IgM anti-DNA antibody-producing B cells.

Materials and Methods

Mice and Generation of Hybridomas. (NZB × NZW)F1 mice were purchased from Harlan/Sprague-Dawley (Indianapolis, IN) and maintained in a pathogen-free environment within the animal facilities at The University of Tennessee, Memphis. Hybridomas were generated as described previously (5). A partial splenectomy and B cell fusion were performed on one mouse. The mouse was completely anesthetized by a combination of intraperitoneal administration of a mixture of xylazine/ketamine/butorphanol (25:40:4 mg/kg body weight) and intermittent inhalation of metaphane throughout the surgical procedure. An incision was made in the left flank. The blood vessels in the vascular bundle that enters the hilus of the spleen were simultaneously sealed and cauterized. The spleen was cut and the free half removed and used to generate hybridomas. The abdominal wall and overlying skin were closed with surgical staples that were removed 10 d after surgery. The remaining half of the spleen was removed at a later date for hybridoma production by the normal procedure.

ELISA for Anti-DNA. The direct-binding, solid-phase ELISA used to detect anti-DNA-positive culture wells after the fusion and determination of IgH and L isotypes have been described in detail (5). Culture wells were chosen for cloning of hybridomas only when the relevant culture supernatant produced a relative antibody activity of 3 on a scale of 1 to 10, with 10 being the maximum activity of the assay (ODmax >1.2). The supernatants were also screened for binding to micro-ELISA plates that were not coated with DNA but were treated identically otherwise. The source of DNA used for screening hybridoma supernatants was commercial calf thymus DNA sheared by sonication. Only hybridomas producing antibody that was positive on DNA-coated plates and negative for binding to plates not coated with DNA were considered for cloning. This screening procedure allowed us to select for hybridomas producing both low- and high-affinity IgM and IgG DNA binding antibodies, as indicated by the data in Table 1. The assay did not discriminate between ssDNA and dsDNA specificity.

cDNA Sequencing. The procedures for isolation of mRNA (16) and cDNA sequencing (17), the sequences of the oligonucleotides used as primers, and the method for sequence comparisons were exactly the same as those we have used previously (7).

Statistical Analyses. The numbers of germline Vh and Vk genes that could encode anti-DNA were estimated from the repeat frequencies of individual Vh and Vk observed among all the different clones represented in this study. Vh and Vk from individual clones were considered to have been derived from the same germline Vh or Vk if the nucleotide sequences were >95% homologous. The identical pair method of Brookes and Carroll (18) was used to obtain the estimates: R = N/A, where N = n(n-1)/2; A = χ2; n = number of dependent sequences; and χ2 = number of sequences derived from the same germline sequence i. The number of dependent sequences (n) used to calculate the estimates was adjusted to account for repeat frequencies among either Vh or Vk sequences that deviated from the expected normal distribution (19). For Vh, n = 42, A = 14, and N = 861; therefore, the estimated number of Vh in the anti-DNA repertoire (R) is 62 (95% confidence interval [CI] = 43, 96). For Vk, n = 55, A = 34, and N = 1,485; therefore, R = 44 (95% CI = 31 and 63). For Vh, n = 28, A = 16, and N = 378; therefore, R = 24 (95% CI = 15 and 36). Calculations of the probabilities that individual Vh or Vk genes, or Vk-Vk combinations, would be expressed at the indicated frequencies in the absence of selection among the population of hybridomas assumed a binomial distribution for any given V gene (20).

Results

Oligoclonality of IgG Anti-DNA Hybridomas. The criteria used in choosing mice for spleen cell fusions to generate hybridomas were age, serum titer, and isotype of spontaneous anti-DNA antibody. Mice used to generate IgM hybridomas were <6 mo of age, with IgG anti-DNA titers <90 and IgM anti-DNA titers >1,000. Mice chosen for generating IgG anti-DNA hybridomas were usually 6–8 mo old or older, with serum IgG anti-DNA titers >1,000. Hybridomas generated from the fusions were chosen for cloning as described in Materials and Methods. This procedure was specifically chosen so as not to bias the population of hybridomas, particularly the IgM, toward either low- or high-affinity antibody. Specificity analyses of the mAbs in competitive ELISAs
confirmed that there was no bias in the selection procedure for hybridomas producing either high- or low-affinity antibody (Table 1).

During the course of this study, 107 anti-DNA antibody-producing hybridomas from 10 different autoimmune (NZB × NZW)F1 mice were generated. Each hybridoma was analyzed for VH and VL cDNA nucleotide sequences (Table 1, and Figs. 1 and 2) and for isotype and DNA specificity of its respective mAb. In each mouse with predominantly IgG serum anti-DNA (mouse nos. 17, 111, 163, 10, 74, 83, and 185), the majority of the IgG hybridomas formed clonally related groups. For the purpose of comparing the clonal repertoire between IgM and IgG anti-DNA antibodies among different individual mice, data in Table 1 and Figs. 1 and 2 are from only one representative hybridoma of a given clonally related group. Clones with more than one hybridoma are indicated with a “c” designation as part of the clone number (e.g., 111-cl). For clones with more than one hybridoma, the number of hybridomas in each clone are indicated in Table 1 and Figs. 1 and 2. Clonal relatedness among relevant hybridomas was confirmed by nucleotide sequences, particularly in the junctional regions between V and D and D and J of the heavy chain, and V and J of the light chain, and by the identity of productive and nonproductive Jv and Jk rearrangements within each clonal member (21). There were 38 IgM hybridomas from 36 clones, and 69 IgG hybridomas from 29 clones (Table 1). Two clones had IgM and IgG hybridomas from the same clone. The degree to which individual clones were expanded in each of the mice was quite variable, from single representatives such as 17s.83 and 163.42 to clones with nine representative hybridomas such as clones 163-cl and 185-cl (Table 1). There was considerable intraclonal variation due to somatic mutation in each of the multiple member clones (“c” clones in Table 1). Detailed analyses of the mutations and their effects on the specificity of the respective antibodies will be presented and discussed elsewhere (N.-T. Jou, D. Tillman, R. Hill, and T. Marion, manuscript in preparation).

Recurrent Vh Gene Usage among Both IgM and IgG Anti-DNA. Multiple clones from two or more animals expressed at least one Vh gene from each of the VH558, Vh7183, VhQ52, and VhS107 germline Vh families (Table 1). For example, 165.14 and 17s.128 each expressed a VH558 family Vh gene that is nearly identical to the previously identified Vh for the anti-DNA hybridoma BXW-DNA16 (Fig. 1 A, BWDNA16) (22). Likewise, 17s-cl and 165.60 had nearly identical Vh that are also similar to VH558-BWDNA16, as did 10-c1 and 17s.166, and 165.72 and 25.12m. Extreme examples of identical or nearly identical Vh gene expression are clones 111.185, 165.27, 165.49, and 17s.83. These clones expressed a VH558 family Vh gene very similar to that expressed by the previously identified anti-DNA hybridoma MLR-DNA22 (Fig. 1 A, DNA22) (22). Eight clones expressed a Vh previously identified for the hybridoma BXW-DNA7 (Fig. 1 A, BWDNA7). In the latter two groups of anti-DNA clones, four clones from three different mice (Fig. 1 A, DNA22) and eight clones from four different mice (Fig. 1 A, BWDNA7), respectively, use the same Vh gene.

The repetitive usage of Vh genes was apparent among both IgM- and IgG-producing clones for each of the Vh genes described above. For example, 111.185 and 165.27 are IgM, and 165.49 and 17s.83 are IgG (Table 1 and Fig. 1 A, DNA22). Likewise, 17p.101, 202.80, 202.38, 202.135, 202.61, 165.3m, and one of the hybridomas in 111-c1 are IgM (Table 1 and Fig. 1 A, BWDNA7). All the hybridomas in 111-c2 and three hybridomas in 111-cl were IgG. Clone 17p-c7 had two IgM hybridomas and one IgG hybridoma with a Vh gene from the Vh10 family (Table 1 and Fig. 3). In almost every case, at least one IgM and one IgG hybridoma expressed the same Vh gene, usually with a different Dk. Notable exceptions were two IgM-producing hybridomas, one of which had a VH derived from the Vh606 family and the other from the Vh36-60 family (Table 1). These two Vh gene families were not represented among any of the IgG-producing hybridomas. Likewise, hybridomas expressing a Vh from the VhS107 family were found only among IgG-producing hybridomas (Table 1 and Fig. 2).

The estimated number of different germline Vh genes that could encode an anti-DNA antibody is 44 (see Materials and Methods). The probability that four different clones out of the total of 63 would express a VH derived from the same germline Vh gene by chance alone is 0.042. These results indicate that among the total population of clones there was preferential expression of VH genes homologous to the VH558 family genes expressed by the anti-DNA hybridomas MRL-DNA22 (111.185, 165.27, 165.49, 17s.83; Fig. 1 A, DNA22) and BWDNA7 (111-c1, 111-c2, 17p.101, 202.80, 202.38, 202.135, 202.61, and 165.3m, 13 hybridomas total; Fig. 1 A, BWDNA7) (22). There was also preferential usage of a VH558-derived Vh gene for which homologous Vh genes have not been reported (Fig. 1 C).

Both the IgM and IgG anti-DNA hybridoma populations independently demonstrated preferential Vh gene expression. The probability that three IgM clones cut out of the total of 36 would express a VH derived from the same germline Vh gene by chance is 0.020. Therefore, there was a strong preference among the IgM hybridomas for Vh genes homologous to genes from the VH558 family (four clones: 165.33, 165.41, 165.52, and 202.17; Fig. 1 C). There was also preferential usage of a gene from the VH558 family similar to the Vh558 gene used by the anti-DNA hybridoma BXW-DNA7 (22) (six clones: 17p.101, 202.80, 202.38, 202.135, 202.61, and 165.3m; Fig. 1 A, BWDNA7). IgM clones 165.60, 165.72, and 25.12m are >92% homologous and use a VH558 family gene homologous to the previously described Vh, for the anti-DNA hybridoma BXW-DNA16 (22).

As stated in the previous section, most of the hybridomas producing IgG anti-DNA was found to be members of clones represented by two or more hybridomas. Of the 29 clones represented among the 69 IgG hybridomas, 18 were represented by two or more hybridomas. If the analysis of preferential Vh gene usage is determined on the basis of the frequency that a particular Vh gene is used among IgG clones, there was preferential expression (p < 0.025) of a Vh gene homologous to the VH558 Vh31 germline gene (17s-c2, 165.3, and 74-c1; Fig. 1 A, S57[Vh31]); and the VhS107 family Vh11.
| Mouse | Clone | Isotype | VH          | VL          | DNA binding specificity* |
|-------|-------|---------|-------------|-------------|--------------------------|
| 17    | p101  | IgM     | V_{m}558    | V_{k}1      | 8.84 NI                |
|       | p73   | IgM     | V_{7183}    | V_{k}21     | >10 NI                  |
|       | p3    | IgM     | V_{m}606    | V_{k}2      | EB* NI                  |
|       | s93m  | IgM     | V_{7183}    | V_{k}1      | 1.39 15% NB            |
|       | s128  | IgM     | V_{m}558    | V_{k}21     | 7.43 19                |
|       | s13   | IgM     | V_{m}558    | V_{k}1      | 0.46 6.51 NB           |
|       | s166  | IgM     | V_{m}558    | V_{k}12     | 0.31 13                |
|       | s-c6(2) | IgM | V_{m}558 | V_{k}4      | 0.05 NI                |
|       | ps-c7(3) | IgM/G2a | V_{10} | V_{k}1      | 0.40 14                |
|       | s-c1(4) | IgG2b | V_{m}558    | V_{k}5      | 0.05 0.12              |
|       | s-c2(2) | IgG2a | V_{m}558    | V_{k}1      | 0.04 NI                |
|       | s-c3(2) | IgG2a | V_{m}558    | V_{k}1      | 0.60 5.78              |
|       | s-c4(3) | IgG2a | V_{m}558    | V_{k}21     | 0.04 0.82              |
|       | s-c5(3) | IgG2b | V_{m}558    | V_{k}1      | 0.47 1.05              |
|       | s83   | IgG1    | V_{m}558    | V_{k}1      | 0.58 NI                |
|       | s2    | IgG2a   | V_{7183}    | V_{k}32     | 7.18 NI                |
|       | s5    | IgG2b   | V_{7183}    | V_{k}19     | 0.34 3.00              |
|       | s130  | IgG1    | V_{7183}    | V_{k}19     | 0.27 1.53              |
|       | s145  | IgG2b   | V_{m}Q52    | V_{k}2      | 0.14 35%               |
|       |       |         |             |             |                         |
| 111   | 68    | IgM     | V_{m}558    | V_{k}1      | EB EB                  |
|       | 185   | IgM     | V_{m}558    | V_{k}1      | 0.36 17%               |
|       | c1(4) | IgM/G2a | V_{m}558    | V_{k}8      | 0.91 0.97              |
|       | c2(3) | IgG2a   | V_{m}558    | V_{k}2      | 0.13 NI                |
|       | 55    | IgG2a   | V_{7183}    | V_{k}1      | 0.25 16%               |
|       | 67    | IgG2a   | V_{7183}    | V_{k}5      | 2.50 16%               |
|       | 33    | IgG2a   | V_{m}S107   | V_{k}1      | 1.13 16% <2           |
|       | 163   | c4(2)   | IgM    V_{7183} | V_{k}1      | 1.55 1.09              |
|       | 42    | IgM    V_{7183} | V_{k}Ox1  | V_{k}1      | 1.30 NB                |
|       | 72    | IgM    V_{m}558 | RGGITTV   | V_{k}5      | 1.53 334               |
|       | 100   | IgM    V_{m}558 | RLRWA     | V_{k}8      | 2.08 NI                |
|       | c1(9) | IgG2b/G2a | V_{7183} | HYYGSRTY   | V_{k}8      | 0.28 1.37              |
|       | c2(3) | IgG2a   | V_{m}S107   | DpygRTRs   | V_{k}10     | 0.6 0.62               |
|       | c3(2) | IgG2a   | V_{m}Q52    | KGLRRAG    | V_{k}2      | 0.5 0.12               |
|       | 47    | IgG2b   | V_{m}558    | GI          | V_{k}9      | 0.6 0.17               |
|       | 165   | 6      | IgM    NA   | NA         | V_{k}-23     | 26% 3.4                |
|       | 3m    | IgM    V_{m}558 | DPPLLRLYY | V_{k}-19    | 0.11 NI                |
|       | 5     | IgM    V_{m}558 | EGCY      | V_{k}-8     | 2 NI                   |
|       | 33    | IgM    V_{m}Q52 | YYYYGSPLN | V_{k}-1     | EB NI                  |
|       | 41    | IgM    V_{m}Q52 | YDGYY     | V_{k}h     | EB EB                  |
|       | 52    | IgM    V_{m}Q52 | YHSTAPWW  | NA*        | EB NI                  |
|       | 54    | IgM    V_{m}36-60 | SGRGA    | V_{k}-23    | 0.11 15%               |
|       | 27    | IgM    V_{m}558 | DG         | V_{k}-1     | 0.095 NI               |
|       | 45    | IgM    V_{m}558 | EA         | V_{k}-8     | 17% NI                 |

continued

166 | Summary of Variable Region Structures and DNA Specificity for Monoclonal Anti-DNA Autoantibodies

| Mouse | Clone | Isotype | VH          | VL          | DNA binding specificity* |
|-------|-------|---------|-------------|-------------|--------------------------|
|       |       |         |             |             |                         |
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**Table 1. (continued)**

| Mouse | Clone¹ | Isotype | VH | VL | DNA binding specificity* |
|-------|--------|---------|----|----|---------------------------|
|       | (continued) |         | V₅ | D₅ | Jᵥ | V₆ | Jₓ | ssDNA | dsDNA | CDLP** |

| 60    | IgM    | V₅558  | GETTVVGKGY | 2 | V₆K-23 | 1 | NI | 8.7 | NB   |
| 3     | IgG1   | V₅558  | GDDLWLRRL    | 2 | V₆K-8 | 5 | 0.14 | 34% | 40   |
| 14    | IgG1   | V₅558  | RYYGREGY    | 2 | V₆K-10 | 4 | 0.16 | NI  | NB   |
| 49    | IgG2a  | V₅558  | RAWD     | 1 | V₆K-8  | 1 | 0.16 | NI  | NB   |
| 202   | 9      | IgM    | V₃Q52   | YYYGSS | 4 | V₆K-8 | 2 | 8.46 | 29% | NB   |
| 17    | IgM    | V₃Q52  | YSDYYGSS | 1 | V₆λ-2 | 2 | NI  | NI  | NB   |
| 33    | IgM    | V₇183  | SRLWLRVG | 1 | V₆K-9 | 4 | NI  | 5.87 | NB   |
| p38   | IgM    | V₇183  | QGWDR | 4 | V₆K-21 | 1 | 3.47 | NI  | NB   |
| 54    | IgM    | V₅558  | LP     | 1 | V₆K-9 | 2 | 0.16 | 17% | NB   |
| 61    | IgM    | V₅558  | LIYYGSI | 3 | V₆K-Ox1 | 5 | 0.29 | NI  | NB   |
| 80    | IgM    | V₅558  | RGYYYGSS | 4 | V₆Kᶠ | 1 | EB  | NI  | 1    |
| s38   | IgM    | V₅558  | GGRYDL | 4 | V₆K-1 | 4 | 0.22 | NI  | NB   |
| 135   | IgM    | V₅558  | GYYGSYS | 3 | V₆K-8 | 2 | 1.97 | NI  | NB   |
| 105   | IgG2a  | V₅558  | RYYRR | 4 | V₆K-9 | 2 | 2.23 | 2.12 | <2   |
| 10    | c1(4)  | IgG    | V₅558  | GDRTG | 2 | V₆K-1 | 1 | 0.70 | 3.17 | 3.5  |
| 25    | 12m    | IgM    | V₅558  | GRYT  | 3 | V₆K-1 | 2 | 0.34 | NI  | NB   |
| 74    | c1(2)  | IgG    | V₅558  | EDWDGG | 3 | V₆K-5 | 2 | 0.09 | 0.14 | 1    |
| c2(2) | IgG    | V₅S107 | DKGRYGA | 1 | V₆K-21 | 1 | 0.5 | 21% | 0.005 |
| 83    | c1(3)  | IgG    | V₇183  | GGTR  | 3 | V₆K-19 | 4 | 1.91 | 2.74 | <0.05 |
| 185   | c1(9)  | IgG    | V₅Q52  | NTPLGRRY | 2 | V₆K-12 | 1 | 0.30 | 0.39 | NB   |

* The DNA binding specificity for ssDNA and dsDNA is presented as the amount (µg/ml) of either ssDNA or dsDNA that was required to produce 50% of maximum binding in the competitive ELISA. Numbers in italics represent values extrapolated from the inhibition curve. Percentages in italics are the maximum percentage inhibition of binding produced by 10 µg/ml competitor. The standard deviation of triplicate wells at each competitor dilution was always <10% of the mean OD₄₀₅. For clones with multiple hybridomas, the data presented are from one representative mAb. NI = no inhibition. EB = enhanced binding; in the presence of the competitor, binding to the solid-phase DNA was increased.

¹ Clones represented by a single hybridoma are designated with a number. Clones with two or more members are designated by a "c" followed by a number. The number of individual hybridomas isolated and analyzed from a clone is in parentheses after each clone number. The "p" and "s" designations of the clones from animal 17 refer to the partial fusion from which the hybridomas were obtained (see text). "p" refers to the first fusion and "s" to the second.

² Nomenclature according to Brodeur and Riblet (70).

³ Amino acid sequence for that part of CDR3 contributed by D₅.

⁴ Nomenclature according to Potter et al. (71).

⁵ CDLP binding is presented as a ratio obtained by dividing the titer of mAb supernatant that produces 50% of maximum binding to CDLP in the solid-phase ELISA divided by the titer that produces 50% of maximum binding to DNA in the solid-phase ELISA for DNA binding. The dilution of supernatant that produced 50% maximum binding to solid-phase DNA was the same dilution used in each respective competitive ELISA for that supernatant. NB = no binding.

Germline gene (163-c₂, 74-c₂, and 111.33; Fig. 1 D). If the estimation of preferential V₅ gene usage is based on the number of hybridomas rather than the number of clones, preference in V₅ gene usage was even stronger (p <0.004). Seven hybridomas expressed a V₅ homologous to V₅558-BWDNA7 (111-c₁ and 111-c₂; Fig. 1 A, BWDNA7). 9–12 hybridomas expressed a V₅ homologous to V₅Q52-165.33 (185-c₁, 17s.145, and 163-c₃; Fig. 1 C); and 12 hybridomas expressed a V₅ homologous to V₅7183-Vh283 (163-c₁ and 85-c₁; Fig. 1 B, Vh283). The latter estimate assumes that the precursors to individual hybridomas are selected independently by antibody receptor-mediated events regardless of the size of the clone of which the respective hybridomas might be members. Although the V₅558 family V₅ gene expressed by the anti-DNA hybridoma 3H9 (Fig. 1 A, 2F2/3H9) was not preferentially expressed among the hybridomas analyzed.

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| 165.14  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
|---------|------------------------|-----------------------------|----------------------------|---|
| 165.56  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.14  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.56  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.14  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.56  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.14  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.56  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.14  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |
| 165.56  | V---P                   | T---Y                        | QSGYVQGTYDV               | 1 |

Figure 1. Translated amino acid sequences for VH (one-letter code). The sequences are grouped according to the V\alpha family from which each VH was derived. Within the VH grouping, sequences are ordered according to homology with the reference sequence. Dashes indicate identity with the reference sequence; blanks indicate either that the VH does not have an amino acid at that position or sequence was not obtained for that region.
An "x" indicates sequence ambiguity for the relevant position. The sequences are numbered according to Kabat et al. (42); CDR regions are enclosed within rectangular boxes. The clone numbers of IgM-producing hybridomas are underlined. The nucleotide sequences from which these translated in this study, this V< sub>n> gene was found to be preferentially expressed among anti-DNA hybridomas from MLR lpr/lpr mice (9). There was no apparent preferential expression of a particular J< sub>n> or V<sub>n>-J< sub>n> combination among either IgM or IgG hybridomas.

**Arginines in VH CDR3 Derived by Unusual D<sub>n> Recombination.** More than 50% of the clones, 35 of 63, had a VH with one or more arginines in CDR3 (Table 1 and Fig. 1). This is a highly characteristic feature of anti-DNA antibodies in mice (6, 7, 9, 22-29) and humans (30, 31). This region of the heavy chain is formed by recombination of one or more D<sub>n> genes within the 5' end of a J<sub>n> gene and the 3' end of a V<sub>n> gene (32, 33). Arginines are generally rare in CDR3 (9) and are the products of random events in the recombination process (24). As we have demonstrated previously (7), arginines in VH CDR3 can be generated by several different mechanisms. These include N sequence addition (34, 35), such as in 17p.73, 17s.93m, 163.100, and 202.105. A shift from the normal reading frame, RF1 (36, 37), of a D<sub>FR1> OR D<sub>SR2> gene to a different reading frame, RF3, can also generate arginines in VH CDR3. This frame shift can be caused either by N sequence addition, such as in 163.100 and 165.3 or D<sub>n>-D<sub>n> recombinant (33) or inversion of a D<sub>n> gene during recombination, such as in 17s.13. All these mechanisms for D region diversity have also been reported by others (9, 24, 38-40).

**Selected V<sub>k> Gene Expression among Both IgM and IgG Hybridomas.** V<sub>k> from each of the V<sub>558>, V<sub>2>, V<sub>4>, V<sub>5>, V<sub>8>, V<sub>9>, V<sub>10>, V<sub>12>, V<sub>19>, V<sub>20>, V<sub>21>, and V<sub>23> gene families and from the V<sub>2>, V<sub>5>, V<sub>RF>; V<sub>1>, and V<sub>2> germline V<sub>k> genes were expressed among the hybridomas analyzed in this study (Fig. 2). The estimated number of different germline V<sub>k> genes that could be used to generate an anti-DNA antibody is 62 (see Materials and Methods). Based upon this number of V<sub>k> genes, the probability that three clones would express the same V<sub>k> gene is <0.018. Therefore, there was preferential usage of a V<sub>k> gene derived from each of the V<sub>2> and V<sub>5> germline families. The preferentially used V<sub>2> gene was 92% homologous to the V<sub>2> gene expressed by the previously identified anti-DNA hybridoma BXW-DNA14 (17p.3, 111-c2, and 17s.145; Fig. 2 C) (22). The preferentially used V<sub>5> gene was 92% homologous to the V<sub>5> gene expressed by the anti-DNA hybridoma MRL-DNA22 (163.72, 17s-c1, and 74-c1; Fig. 2 D) (22). Preference for V<sub>k> derived from the V<sub>1> and V<sub>8> families was much stronger (p <0.000080) for seven or more clones expressing the same (95% homologous) V<sub>k> gene among IgM hybridomas and among IgG clones (Fig. 2 A). Preference for V<sub>k> expressed by the previously reported anti-DNA hybridoma MRL-DNA4 (22). Most of the clones expressing a V<sub>k> gene from the V<sub>8> group expressed a V<sub>8> gene 98% homologous to the V<sub>k> gene expressed by the anti-DNA hybridoma DNA5 from clone 4 (7).

The preference for V<sub>1> and V<sub>8> was apparent among both IgM and IgG hybridomas (p <0.0027 and p <0.00013, respectively). Among the IgM clones, five clones (17s.93m, 17p.101, 25.12, 111.185, and 165.27; Fig. 2 A) each expressed a VL derived from the same V<sub>1> gene, and three clones (202.9, 165.45, 111-c1, and 165.5; Fig. 2 B) each expressed a VL derived from the same V<sub>8> germline gene. Among IgG clones, four expressed a VL derived from the same V<sub>1> germline gene (17s-c2, 17s-c3, 17s-c5, and 17s.13; Fig. 2 A), and four expressed a V<sub>k> derived from the same V<sub>8> germline gene (165.49, 163-c1, 111-c1, and 165.3; Fig. 2 B). These results indicate preferential clonal selection and expansion of anti-DNA B cells expressing either V<sub>1> or V<sub>8> during both the early IgM and late IgG anti-DNA response in the autoimmune mice from which our panel of hybridomas was obtained.

**J<sub>1> Functional Diversity among VL of Anti-DNA Hybridomas.** Assuming that each J<sub>1> has an equal probability for expression among anti-DNA B cell clones, J<sub>1> was over-represented and J<sub>4> and J<sub>5> were underrepresented among the clones listed in Table 1 (p <0.025). This unequal representation of J<sub>1> is consistent with normal J<sub>1> usage in mice (41). However, 8 of the 23 J<sub>1> clones had arginine (CGG) as the first codon encoded by J<sub>1> instead of the germline cryptophan (TGG). This position would correspond to position number 96 in VL CDR3, according to the numbering convention of Kabat et al. (42). This conversion would most likely occur from junctional diversity between V<sub>k> and J<sub>1> (43) but could also be due to somatic mutation. 17 hybridomas from eight clones expressed the altered J<sub>1> (IgM clones 111.185, 17p.3, and 163.72; and IgG clones 10-c1, 111-c1, 163-c2, 17s.5, and 74-c2). Each of the IgG clones had two to nine members, indicating that B cells with VL derived from J<sub>1> with the cryptophan->arginine conversion may have been selectively expanded.

**Selection for V<sub>558,58,558-V<sub>1> and V<sub>558,58,558-V<sub>8>**. Assuming an equal probability for any of the possible anti-DNA V<sub>n> genes to be expressed with any of the possible V<sub>k> genes in a given B cell, significantly more clones expressed V<sub>n>-V<sub>k> combinations generated from a V<sub>558> family V<sub>n> and either a V<sub>1> or V<sub>8> family V<sub>k> than would have been expected if V<sub>n> and V<sub>k> genes were selected randomly (p <0.0018 for V<sub>558,58,558-V<sub>1> and p <0.029 for V<sub>558,58,558-V<sub>8>). When the data were analyzed to determine if a particular V<sub>n> gene-V<sub>k> gene combination was expressed at a higher-than-expected frequency among the clones, one combination was found to be preferentially used. The IgG hybridoma 17.83 and the IgM hybridomas 111.185 and 165.27 expressed nearly identical V<sub>k> and V<sub>k> genes. The V<sub>n> gene was similar (≈93% homology) to the V<sub>558> family V<sub>n> gene previously reported for the anti-DNA hybridoma BXW-
DNA22 (Fig. 1 A, BWDNA22) (22). The V\(_\kappa\) gene was the same (>98% homology) V\(_\kappa\)1 family gene previously reported for MRLDNA4 (Fig. 2 A, MRLDNA4). The probability that this V\(_\kappa\)V\(_\lambda\) combination would have appeared three times by chance among the 63 clones is <0.0002. These results are even more striking since each of the three clones that express the V\(_\kappa\)558-BWDNA22-V\(_\lambda\)-MRLDNA4 combination was obtained from a different animal. Moreover, 111.185 and 165.27 are nearly identical in both VH and VL, including the D\(_\kappa\) encoded VH CDR3. These two hybridomas differed in their V\(_\lambda\) and V\(_\kappa\) sequences by only two identifiable amino acids at positions 94 and 96 in VL CDR3 (Fig. 2 A). The difference in position 96 between the two V\(_\lambda\) sequences is due to the J\(_\lambda\) difference discussed above. 165.27 has the germline tryptophan at this position, while 111.185 has arginine.

**IgM and IgG Anti-DNA Are Structurally Similar.** The data in Table 1 and Figs. 1 and 2 indicate that the IgM and IgG populations are completely overlapping with respect to VH and VL structure. The clones 17s.83, 111.185, and 165.27 described above demonstrate particularly well the similarity in structure between IgM and IgG anti-DNA. In two clones from two different mice, both IgM and IgG hybridomas were obtained from the same clone (Fig. 3). The 17s-c7 clone is particularly interesting. The hybridomas from this mouse are divided into p and s groups. The p group was generated...
from a partial splenectomy of this mouse at the age of 24 wk, when the mouse’s serum IgM anti-DNA titer was 1,800 and IgG anti-DNA titer <90. Five IgM anti-DNA hybridomas out of a total of five were analyzed from this fusion (Table 1). The remaining half of the spleen was removed 1.5 mo later when the mouse’s serum IgG anti-DNA titer was >2,400. Six IgM and 20 IgG hybridomas out of a total of 94 were analyzed from this fusion. The IgG hybridoma 17.5.161 derived from the second fusion was found to be clonally related to IgM hybridomas 17p.79 and 17p.80 obtained from the first fusion (Fig. 3A). Clonality of these hybridomas was confirmed by identity of both productive and nonproductive Jδ and Jε rearrangements (21) (not shown). The IgM hybridoma 111.19 and the IgG hybridomas 111.61, 111.100, and 111.109 from clone 111-c1 were also found to be clonally related, although these hybridomas were all generated in the same fusion (Fig. 3B). Somatic mutations were apparent in both clones. The effects of these mutations on specificity for DNA will be discussed elsewhere (Jou et al., manuscript in preparation). These results indicate that some, if not all, of the secondary, IgG anti-DNA antibodies in autoimmune (NZB x NZW)F1 mice.

Structural Basis for ssDNA vs. dsDNA Specificity and Selection for dsDNA Specificity among Clonally Expanded B Cells.
The mAb produced by each hybridoma was assayed for specificity of binding to ssDNA, dsDNA, and cardiolipin (CDLP) (Table 1). Although all the mAbs bound to DNA in a direct ELISA (Materials and Methods), a small number (8 IgM mAbs out of 107 total mAbs) were not competitively inhibited by either ssDNA or dsDNA in the competitive ELISA. This may be a reflection of the higher avidity of these antibodies for binding to immobilized DNA. In fact, six of
the IgM mAbs (17p.3, 111.68, 165.33, 165.41, 165.52, and 202.80) that were not competitively inhibited demonstrated increased binding to the solid-phase DNA in the presence of competitor DNA (Table 1). The most likely explanation for this observation is that these antibodies have relatively low affinity for binding to DNA. In the presence of competitor and because of the high avidity of IgM, the antibodies are able to form a lattice that stabilizes their binding to the immobilized DNA. There was no significant relationship between particular 

\[ V_\alpha \text{ or } V_\delta \text{ gene expression by different hybridomas and the absolute ability of the antibodies from those clones to bind to ssDNA vs. dsDNA or CDLP (0.05 < p < 0.10). Likewise, there was no correlation between arginines in VH CDR3 and specificity for DNA among the antibodies. However, as discussed below, the position of arginines in VH CDR3 had a remarkable effect on the DNA specificity of the respective mAb; and particular VH and VL structural combinations also had demonstrable effects on DNA specificity.

All of the mAbs that had an arginine at position 96 in VL bound to dsDNA. As indicated above, arginine at this position was most likely derived from junctional diversity in 

\[ V_\gamma \text{ to } J_\gamma \text{1 recombination. The recurrent nature of this randomly generated structure among clones using } J_\gamma \text{1 suggests that this structure may have been specifically selected. This possibility is supported by results from a comparison of mAbs 111.185 and 165.27. Although both mAbs bind to ssDNA very well (Table 1), 111.185 binds poorly but measurably and consistently to dsDNA. These two antibodies differ in VH and VL at only two identifiable positions. 111.185 has an isoleucine instead of valine at position 94 in VL CDR3. More importantly, 111.185 has an arginine instead of tryptophan at position 96 in VL CDR3.

There was a very interesting and highly significant trend relative to the specificity of antibodies produced by IgM vs. IgG clones. A significantly higher percentage of IgG clones produced antibody that bound to both ss- and dsDNA or dsDNA alone as opposed to ssDNA binding alone (Table 1). Of 27 IgG clones, 21 had one or more hybrids that produced anti-dsDNA antibody, compared with 12 of 34 IgM clones (p < 0.001). This difference was even more pronounced when individual hybridomas that did not clone than those clones were compared (p < 0.0005). The difference in dsDNA binding between IgM and IgG mAbs was also observed within a single clone, 17p.s-c-7. The IgM mAbs 17p.79 and 17p.80 both bound to ssDNA with relatively high avidity but did not bind to dsDNA (Jou et al., manuscript in preparation). The IgG mAb from the clonally related hybridoma 17s.161, obtained 1.5 mo later, bound to ssDNA and dsDNA.

Discussion

The results from our analyses of the hybridomas described above confirm our previously reported results that the IgG anti-DNA hybridomas from an individual (NZB × NZW)-F1 mouse were oligoclonal and somatically mutated (6, 7), and therefore had the characteristics of secondary immune antibodies (21, 44–48). The present results indicate that the oligoclonality of IgG anti-DNA hybridomas observed previously was not unique to a single mouse and may, in fact, be a general characteristic of IgG anti-DNA hybridomas obtained from individual (NZB × NZW)-F1 mice. These results corroborate the hypothesis that the spontaneous IgG anti-DNA antibody response characteristic of these mice is generated as a clonally selective, Ig receptor–specific immune response. IgG anti-DNA hybridomas from MRL lpr/lpr mice are also oligoclonal and the mAbs from those hybridomas are characteristic of secondary immune antibodies (8, 9, 25).

Oligoclonality of IgG anti-DNA antibodies has also been observed among anti-DNA mAbs from autoimmune (NZB × SWR)-F1 mice (27) and among V\_11 encoded anti-DNA in (NZB × NZW)-F1 mice (28).

The IgM anti-DNA hybridomas from the mice in this study also appear to have been derived from B cells that were stimulated in a clonally selective, antigen-specific manner. This conclusion is based on the statistical analyses of 

\[ V_\alpha \text{ and } V_\gamma \text{ gene expression among the IgM clones. Even though each of the 44 } V_\alpha \text{ and 62 } V_\gamma \text{ germline genes in the estimated anti-DNA V gene repertoire could encode an anti-DNA antibody, two } V_\alpha \text{ and two } V_\gamma \text{ genes were each expressed in a much larger number of IgM hybridomas than would have been expected in the absence of antibody receptor–mediated selection. The preferential V gene expression among IgM hybridomas was not the end result of the process by which hybridomas were chosen for cloning since the only criterion used was the ability of the antibody in the respective fusion well to bind DNA. Based upon the results from the competitive ELISA used to test the specificity of each mAb (Table 1), the selection of hybridomas for cloning was not biased with respect to the specificity or affinity of the respective mAbs. As the results in Table 1 clearly demonstrate, hybridomas producing both high- and low-affinity mAbs were represented in our sample. We (4) and others (49) have demonstrated that the hybridomas obtained from autoimmune mice are representative of the serum antibody in the fusion donor.

This report provides the first demonstration that both IgM and IgG anti-DNA antibodies within individual autoimmune mice may be derived from the same B cell clones. The results demonstrate that the V regions of IgM and IgG anti-DNA antibody in autoimmune (NZB × NZW)-F1 mice are structurally similar. Also, the 

\[ V_\alpha \text{ and } V_\gamma \text{ genes that were preferentially expressed among the IgM anti-DNA hybridomas were also preferentially expressed among the IgG hybridomas. Within individual mice, both IgM and IgG anti-DNA antibodies may be produced by clonally related B cells. Therefore, B cells that are selectively stimulated to produce the IgM anti-DNA seen early in autoimmune (NZB × NZW)-F1 mice may differentiate and clonally expand to generate the IgG anti-DNA seen later in the same mice. In addition, the results demonstrate that there was greater specificity for dsDNA binding among the IgG mAbs than the IgM. This may indicate preferential selection for dsDNA specificity among B cell clones that are stimulated to expand and differentiate to IgG production. The results suggest that the stimulus
for this B cell clonal expansion may be native DNA. The results are also consistent with and may explain the observed progression of anti-DNA autoantibodies in both mouse and human lupus from more ssDNA-specific to more dsDNA-specific antibody (1, 3). Taki et al. (50) have likewise isolated an IgG hybridoma from an (NZB × NZW)F1 mouse that may be clonally related to an IgG hybridoma from the same mouse. The IgG mAb had much higher avidity for dsDNA than the IgM mAb. The VH used by both hybridomas was the same, and H chain rearrangements in the two hybridomas were the same. However, since the authors did not analyze the respective hybridomas for either L chain sequences or rearrangements, the clonal relatedness of the two hybridomas cannot be assured.

Results from previous studies of B cell activity and antibody production in autoimmune (NZB × NZW)F1 mice led to the hypothesis that anti-DNA antibody was a byproduct of polyclonal B cell activation (reviewed in reference 14). In light of the results from analyses of the effect of the host environment in which B cells develop on the ontogeny of anti-DNA autoantibody (15, 51) and the clonotypic analyses of IgG anti-DNA antibodies in autoimmune mice (6, 8), this hypothesis has recently been modified to propose that the early, IgM anti-DNA B cells are polyclonally activated and that antigen-specific (DNA) selection occurs subsequent to this event (14). This process would yield IgM anti-DNA with the structural characteristics of a randomly selected population of DNA-specific antibodies and IgG anti-DNA with the structural characteristics of a clonally selected population of antibodies. Our results are not consistent with this hypothesis. Although the results cannot exclude the existence of a population of polyclonally activated B cells in the (NZB × NZW)F1 mice used in this study, they suggest that both the IgM and IgG anti-DNA hybridomas were derived from B cells stimulated by antibody receptor-specific events. As discussed below, those events were probably antigen-specific stimulation, most likely by DNA or complexes containing DNA.

Previous analyses of V gene diversity among autoimmune anti-DNA antibodies have demonstrated preferential expression of Vh genes from the Vh558 family (9, 25, 52). Radic et al. (25) estimated that 15 Vh558 genes may encode anti-DNA (95% CI = 9, 42). Using the identical pair method (18), we estimated the number of Vh558 genes that could encode anti-DNA to be 24 (95% CI = 15, 36), which agrees reasonably well with the previous estimate. The recurrence of particular Vh genes among the anti-DNA hybridomas analyzed in this study is striking particularly when compared with the Vh gene expression seen among anti-DNA hybridomas in previously published studies. A summary of the recurrence of Vh and Vk genes among all previously published spontaneous anti-DNA antibody variable region sequences is presented in Tables 2 and 3. The Vh genes that were preferentially expressed among the hybridomas sequenced in this study have been recurrent among the anti-DNA antibodies sequenced in other laboratories. Moreover, even the Vh genes that were not preferentially expressed among our population of hybridomas are recurrent among the total murine population of anti-DNA antibodies for which VH sequences have been published (Table 2). Preferential expression of Vh genes that has not previously been apparent among anti-DNA (9, 25) could be identified here, because of the larger number of sequences analyzed in this study (Fig. 2). Those Vh genes that are preferentially expressed among the hybridomas in this study are recurrent among the total population of mouse spontaneous anti-DNA for which VL sequences have been reported (Table 3). Like the Vh genes, even those Vk genes that were not preferentially expressed among our population of hybridomas are recurrent in the total population of mouse spontaneous anti-DNA. The Vh558-Vk1 and Vh558-Vk8 combinations that were preferentially expressed among the hybridomas analyzed in our studies have also been observed among previously analyzed anti-DNA hybridomas (7–9, 24).

The recurrent nature of particular Vh-Vk combinations and individual Vh and Vk genes, as described above, is indicative of a strong preference for these variable region structures among anti-DNA hybridomas. The rather large number of Vh and Vk genes that can potentially generate an anti-DNA antibody indicates that there is no Vh or Vk gene restriction for the generation of these antibodies. Even the most highly represented Vh and Vk genes were found in only 13% and 11% of the clones, respectively. Rather, the recurrent and preferential use of particular Vh-Vk combinations is more consistent with clonal selection of anti-DNA B cells through antibody receptor-mediated events. As discussed below, the most likely event to control such selection is antigen-specific selection by DNA or DNA complexes. The lack of Vh and Vk restriction is consistent with results from previous studies of Vh gene diversity among anti-DNA hybridomas from a individual (NZB × NZW)F1 mouse (10) and from MRL lpr/lpr mice (9). Therefore, as previously discussed in detail (7, 9), shared idiotypic anti-DNA (5, 53–57) is unlikely to result from Vh or Vk gene restriction. Rather, shared idiotypic anti-DNA is more likely related to structures that control the specificity of anti-DNA (9).

All the clones with the junctionally derived arginine in J1 produce anti-DNA antibodies that bind dsDNA (Table 1, and Jou et al., manuscript in preparation). Moreover, this same J1 arginine has appeared in the anti-DNA hybridoma DNA4 and the clonally related hybridomas DNA5, DNA6, and DNA7 from our laboratory, and among anti-DNA hybridomas from other laboratories: B62 (29) and BxW-DNA14 (22) from (NZB × NZW)F1; 13 and 30 from (SWR × NZB)F1; no. 7 (27); and 2B11 from BALB/c (58). All the monoclonal anti-DNA with J1 that have arginine at position 96 bind dsDNA. These results suggest that arginine at position 96 in VL CDR3 may be a highly selected structure, especially for dsDNA binding. The canonical L chain for IgCκ antiarsonate antibodies also has an arginine at position 96 in VL CDR3 that appears to be generated from junctional diversity (59). This VL structure is highly selected among antiarsonate antibodies. Arginines in both VH CDR3 and J1 from the same mouse. The IgG mAb had much higher avidity for dsDNA than the IgM mAb. The VH used by both hybridomas was the same, and H chain rearrangements in the two hybridomas were the same. However, since the authors did not analyze the respective hybridomas for either L chain sequences or rearrangements, the clonal relatedness of the two hybridomas cannot be assured.

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Table 2. Recurrent \( V_\text{n} \) Gene Usage among Spontaneous Anti-DNA Autoantibodies

| \( V_\text{n} \) gene group* | Relevant clones from Fig. 1† | Hybridomas or clones from other laboratories§ | Reference |
|-----------------------------|-----------------------------|---------------------------------------------|-----------|
| \( V_\text{n}588\text{-}BWDNA16 \) | 165.14, 17s.128 | BXW-DNA16 | 22 |
|                             | 17s-c1, 165.60 | A52 | 24 |
|                             |                | C72 | 29 |
|                             |                | BV16-19 | 26 |
|                             |                | Clones H and I, 82-3 | 25 |
|                             |                | H130 | 23 |
|                             |                | A6.1 | 67 |
| \( -2F2(3H9) \) | 17s.c4, 17s.13 | Clones A, B, and E; D20 | 9 |
|                             |                | Clone 4 | 7 |
| \( -\text{MRLDNA22} \) | 111.185, 165.27, 165.49, 17s.83 | MRL-DNA10, MRL-DNA22 | 22 |
|                             | 163.100 | 564, 550, 567, 563 | 27 |
|                             |                | 05, 11, 12 | |
| \( -\text{BWDNA7} \) | 111-c1, 111-c2, 17p.101 | BXW-DNA7 | 22 |
|                             | 202.80, 202.s38, 202.135 | 8-1, D30 | 25 |
|                             | 202.61, 165.3m | D444, D44 | 24 |
|                             |                | BWR4 | 29 |
| \( -\text{SS7(\text{Vh31})} \) | 17s-c2, 165.3, 74-c1 | Clones C and F | 9 |
| \( \text{Vh7183-Vh283} \) | 163-c1, 83-c1 | Clone D, DP12, S106 | 9 |
|                             |                | 33-2 | 25 |
| \( -\text{DNA13} \) | 17.s130 | Clone 3, DNA3, DNA4 | 7 |
| \( \text{VhS107-Vh11} \) | 163-c2, 74-c2, 111.33 | D42 | 24 |
|                             |                | B62 | 29 |
|                             |                | DP1 | 9 |
|                             |                | Clones 1-7 | 28 |
| \( \text{VhQ52} \) | 163-c3 | D23 | 84 |
|                             |                | 9-15, 9-4 | 50 |
| \( \text{Vh10} \) | 17ps-c7 | MRL-DNA4 | 83 |
|                             |                | BV04-01 | 26 |

* The \( V_\text{n} \) gene group refers to the reference sequences used in Fig. 1.
† Relevant sequences from Fig. 1 are clones that share the indicated \( V_\text{n} \) gene sequence.
§ The clones or hybridomas indicated (from previous publications indicated by the reference) are homologous to the indicated \( V \) region sequence.

must be a strong selection for DNA-specific B cells with antibody receptors that have arginines at these positions.

Because of its basic charge and potential ability to form hydrogen bonds with either G-C base pairs through the major groove or phosphate groups on the backbone of duplex DNA, arginine has been predicted to be important for protein binding to DNA (60). Direct evidence for such a role of arginine in anti-DNA mAb BV04-01 has been demonstrated (61). Therefore, selection for one or more arginines in VH CDR3 and at position 96 in VL CDR3 may occur because arginines at these positions increase the potential for DNA binding by the respective antibodies. Mutations that result in arginine replacements in VH CDR have been demonstrated to have marked effects on the specificity and relative affinity of anti-DNA antibodies (7, 8, 25, and Jou et al., manuscript in preparation). Moreover, the most frequent replacement mutations among anti-DNA antibodies are to arginine or asparagine (25), another amino acid predicted to be particularly impor-
### Table 3. Recurrent $V_t$ Gene Usage among Spontaneous Anti-DNA Autoantibodies

| $V_t$ gene group* | Relevant clones from Fig. 1† | Hybridomas or clones from other laboratories§ | Reference |
|-------------------|-------------------------------|---------------------------------|----------|
| $V_t$1-MRLDNA4    | 17s-c2, 17s-c3, 17s-c5        | MRL-DNA4, MRL-DNA10             | 22       |
|                   | 17s.93, 17s.13                | DP1, 1E81                       | 9        |
|                   | 202.38, 111.68                | BV16-19, BV04-01                | 26       |
| $V_t$8-DNA5       | 202.9, 165.45, 165.49, 163-c1 | Clone 4                         | 7        |
|                   | 111-c1, 165.3, 165.5          | Clone G                         | 9        |
|                   | 202.135                       | D23                             | 84       |
|                   |                               | Group 1                         | 28       |
|                   |                               | A52                             | 24       |
|                   |                               | Clone C, D20                    | 9        |
| $V_t$2-BWDNA14    | 17.p3, 111-c2, 17s.145        | BXW-DNA14                       | 22       |
|                   |                               | B62                             | 29       |
|                   |                               | D23                             | 9        |
| $V_t$4/5-DNA22    | 163.72                        | MRL-DNA22                       | 22       |
|                   |                               | Group 3                         | 28       |
|                   | 17s-c6                        | BWR4                            | 29       |
| $V_t$9-DNA13      | 163.47                        | DNA13                           | 7        |
|                   |                               | 33-2                            | 25       |
| $V_t$10-v-16      |                               | DNA3, DNA4                      | 7        |
|                   |                               | 8-1                             | 25       |
| $V_t$21-$V_t$21E  | 17s-c4                        | 05, 11, 12                      | 27       |
| $V_t$23-DP12      | 165.60, 165.6                 | DP12                            | 9        |
|                   | 165.54                        | Clone I                         | 25       |
|                   |                               | D444                            | 24       |
| $V_t$Ox-1-45.21.1 | 202.61                        | 564, 550, 567, 563              | 27       |
|                   | 163.42                        | BWR5                            | 29       |

* The $V_t$ gene group refers to the reference sequences used in Fig. 2.
† Relevant sequences from Fig. 1 that share the indicated $V_t$ gene sequence.
§ The clones or hybridomas indicated (from previous publications indicated by the reference) are homologous to the indicated $V$ region sequence.

Tant for protein binding to DNA (60). An asparagine in the binding site of mAb BV04-01 has also been demonstrated to participate in the binding of DNA by this mAb (61).

In light of these observations, the comparison of consensus VH and VL CDR sequences generated from all of the mAbs in this study generates very interesting results. To generate these results, all of the mAbs in Table 1, which would include all of the individual members of the multiple member clones (Jou et al., manuscript in preparation), were sorted into one of three categories: loDNA, ssDNA, and dsDNA (Fig. 4). The loDNA category consists of those mAbs that were not competitively inhibited from binding by <2 $\mu$g/ml of either ssDNA or dsDNA in the competitive ELISA. There are 14 IgM and two IgG mAbs in this group. The ssDNA group consists of those mAbs that were competitively inhibited by <2 $\mu$g/ml ssDNA but not dsDNA. There are 15 IgM and 31 IgG mAbs in this group. The dsDNA group consists of those mAbs, five IgM and 33 IgG, that were com-
petitively inhibited by \( \leq 2 \mu g/ml \) dsDNA. Of the 107 mAbs, seven were not included because either the VH or VL sequence was incomplete for each group. Since there were two aligned (42), and consensus CDR sequences for both VH and VL were generated for each group. There were two large clones in the dsDNA group (163-cl and 185-cl), consensus CDR sequences were also separately generated for the dsDNA mAbs according to the criteria described in the text. Determination of CDR regions is according to Kabat et al. (42). The vertically aligned numbers and letters at the top of the figure indicate amino acid positions (42). The VH gene family representation among antibodies in the dsDNA group was 14 V\(_{\gamma}558\), 12 V\(_{\gamma}7183\), 9 V\(_{\gamma}Q52\), and 3 V\(_{\gamma}S107\). The VH composition was 5 V\(_{\gamma}5\); 12 V\(_{\gamma}8\); 8 V\(_{\gamma}12\); 3 V\(_{\gamma}10\); 2 each V\(_{\gamma}9\), V\(_{\gamma}1\), and V\(_{\gamma}2\); and 1 each V\(_{\gamma}1\), V\(_{\gamma}Ox1\), V\(_{\gamma}21\), and V\(_{\gamma}19\).
the binding specificities of the antibodies in the respective groups. Of 20 mAbs with a V\textsubscript{8} VL, 12 bind strongly to dsDNA, while only 1 of 26 mAbs with a V\textsubscript{1} VL binds strongly to dsDNA (Table 1, and Jou et al., manuscript in preparation). Five mAbs with V\textsubscript{5} VL also bind to dsDNA very well (Table 1, and Jou et al., manuscript in preparation).

Of the five V\textsubscript{5} mAbs, four have an arginine at position 29 in VL CDR1 analogous to the V\textsubscript{8} mAbs that bind to dsDNA strongly (Fig. 2, B and D). The mAb from 202.135 and all of those from the hybridomas in clone 111-c1 have V\textsubscript{5}58-B\textsubscript{7}DNA\textsubscript{7} and V\textsubscript{8}-DNA\textsubscript{5} (Figs. 1 A and 2 B). The VH CDR3 for both clones are similar as well. The mAbs from 111-c1 all bind dsDNA (Jou et al., manuscript in preparation), whereas the mAb from 202.135 only binds to ssDNA. Given the above results, the difference in VL CDR1 position 29 between these two clones may contribute in part, if not totally, to the difference in DNA binding by these mAbs, although there are other sequence differences between these two clones. A notable position in the consensus for dsDNA-binding mAbs with VH from the V\textsubscript{5}58 family is position 96 in VL CDR3. The consensus for this group at that position is an arginine. As detailed above, arginine at this position is probably generated by junctional diversity in the first codon of J\textsubscript{1}.

The VH and VL CDR sequences for the mAbs from clone 163-c1 are nearly identical to the consensus sequences for V\textsubscript{7}183 dsDNA-binding antibodies (Fig. 4). Of nine mAbs from this clone, eight bind to dsDNA with relatively high avidity (Table 1, and Jou et al., manuscript in preparation). A computer model of one of the mAbs from clone 163-c1, 163.1, was generated using the crystallographic coordinates for the antilysozyme mAb HyHEL5 (62) (Fig. 5). The hypothetical binding of a model of poly(dA-dT)-poly(dA-dT) to the antibody-combining site was obtained by docking the DNA with the antibody-combining site. This DNA forms a normal B form double helix. The amino acids predicted by the computer model to be in positions to form hydrogen bonds with either base pair or backbone structures of the DNA are identified in Fig. 5. As the model illustrates, lysine at position 50, serine at position 52a, and arginine at position 100 in the heavy chain, and arginine at position 29 and asparagine at position 31 in the light chain, are each in a position that would allow them to form hydrogen bonds with phosphate oxygens on the DNA backbone. The arginines in VH position 100 and VL position 29 correspond to residues that were predicted by the dsDNA group consensus sequence (Fig. 4) to be important for dsDNA binding. Among the eight hybridomas clonally related to 163.1, a shared somatic mutation at VH position 50 and a unique mutation at VH position 52a can be directly correlated to differences in dsDNA binding by the respective mAbs (Jou et al., manuscript in preparation). Therefore, the amino acids predicted by the hypothetical model to be responsible for DNA binding by mAb 163.1 are the same ones that have been predicted to be important for dsDNA binding by structural and serological analyses of the mAbs from clone 163-c1.

The pathogenetic potential of the mAbs described in this report to initiate autoimmune disease has not been directly tested. O'Keefe et al. (63) have used the criterion of variable region cationicity, characteristic of antibodies expressing the Id564 idiotype, as an indicator of pathogenicity. Based upon this criterion, many of the mAbs described here and certainly the antibodies that bind to dsDNA with relatively high avidity would be expected to be pathogenic, as would most of the dsDNA-binding antibodies that have been reported by others, such as A52 (24), mAbs from clone A (9), and mAbs from clone H (25). This criterion is certainly consistent with the original observations of Ebling and Hahn (64) and Dang and Harbeck (65) that the anti-DNA antibodies deposited in nephritogenic mouse kidneys are cationic. The selection for basic amino acids within the V regions of most anti-DNA antibodies demonstrated here and by others (6, 7, 9, 24, 25, 29) may account for the cationicity of pathogenic anti-DNA antibodies. Of those anti-DNA mAbs that have been directly demonstrated to initiate or accelerate nephritis, H130 (66), A6.1, and 3GB3 (67) have a neutral pI. Therefore, determination of pathogenicity among anti-DNA mAbs cannot be related only to pI or idiotype expression (66, 67). Moreover, the formation of glomerular immune deposits by the mAbs H130, H241 (66), and A52 (68) were independent of DNA binding by the mAbs. These observations lead to the interesting hypothesis that the production of anti-DNA antibody is induced and sustained by DNA or DNA complexes, but pathogenesis may occur independently of DNA binding. Clearly future experiments will be needed to sort out the structural basis for the pathogenicity of anti-DNA antibody in lupus nephritis.

The most common feature among anti-DNA antibodies as demonstrated here and elsewhere (6-9, 24-27, 29, 69) is the selective expression of VH and VL structures that would be predicted and in some cases can be demonstrated to influence specificity for DNA. Although these results cannot rule out other structurally selective mechanisms such as idiotype networks and DNA crossreactive antigens that may contribute to the generation of anti-DNA, they are most consistent with the hypothesis that anti-DNA originates and is sustained by an antigen-specific immune response to DNA, most likely in a complex with proteins that could provide the necessary source for a T\textsubscript{n} epitope.

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