Molecular Characteristics of Antibodies Bearing an Anti-DNA-associated Idiotype
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Summary
Anti-double-stranded DNA antibodies are the hallmark of the disease systemic lupus erythematosus and are believed to contribute to pathogenesis. While a large number of anti-DNA antibodies from mice with lupus-like syndromes have been characterized and their variable region genes sequenced, few human anti-DNA antibodies have been reported. We describe here the variable region gene sequences of eight antibodies produced by Epstein-Barr virus (EBV)-transformed B cells that bear the 3I idiotype, an idiotype expressed on anti-DNA antibodies and present in high titer in patients with systemic lupus. The comparison of these antibodies to the light chains of 3I+ myeloma proteins and serum antibodies reveals that EBV transformation yields B cells producing antibodies representative of the expressed antibody repertoire. The analysis of nucleotide and amino acid sequences of these antibodies suggests the first complementarity determining region of the light chain may be important in DNA binding and that paradigms previously generated to account for DNA binding require modification. The understanding of the molecular genetics of the anti-DNA response requires a more complete description of the immunoglobulin germ line repertoire, but data reported here suggest that somatic diversification is a characteristic of the anti-DNA response.

Systemic lupus erythematosus (SLE) is an autoimmune disorder with a wide spectrum of clinical manifestations. The glomerulonephritis of SLE is thought to be caused, in part, by antibodies to double-stranded DNA (dsDNA)1 (1-3). Antibodies to dsDNA are unique to this disease. Their presence correlates with disease activity and they can be eluted from the kidneys of patients with lupus nephritis (1). Recent studies have shown that anti-DNA mAbs perfused through kidneys in vitro or injected into mice in vivo can lead to glomerular pathology similar to that seen in SLE (4, 5). Anti-DNA antibodies are thought to cause disease either by binding directly to glomerular basement membrane antigens or by depositing as immune complexes in the glomeruli.

To understand the molecular genetic origin of anti-dsDNA antibodies and the structural basis for DNA binding, we undertook an idiotypic analysis of anti-DNA antibodies. The antibodies reported here all bear the 3I idiotype. This idiotype is present on light chains of anti-DNA antibodies (6). High titered expression of 3I+ antibodies is present in 80% of SLE patients with anti-DNA activity. 3I+ Igs are deposited in the skin and kidneys of SLE patients indicating that 3I+ antibodies include a pathogenic subset (7). Nonetheless, like other autoantibody-associated idiotypes, the 3I idiotype is also present on antibodies without DNA binding specificity (8, 9). From studies on 3I+ myeloma proteins, we have hypothesized that in this idiotype system, DNA binding is acquired by somatic mutation of germ line variable region genes that do not, in their germ line configuration, display specificity for DNA.

Previously, our laboratory has shown that 3I+ B cell lines derived from lupus patients and from a myeloma patient with a 3I+ myeloma protein preferentially utilize members of the V\_K1 gene family to encode their light chains (10). To further characterize 3I+ Igs and attempt to define the structural basis for idiotype and the motifs contributing to DNA binding, we decided to obtain sequence data from a large number of antibodies. The nucleotide and deduced amino acid sequences of eight 3I+ Igs produced by B cell lines from SLE and myeloma patients are reported. In addition, we report partial amino acid sequence analysis from several 3I+ myeloma proteins and a consensus sequence from polyclonal 3I+ anti-DNA antibodies isolated from the serum of a SLE patient. The mutational analysis of these antibodies is at-

1 Abbreviations used in this paper: dsDNA, double-stranded DNA; R/S, replacement to silent; FW, framework.
Materials and Methods

Generation of Anti-DNA Antibodies. Anti-DNA antibodies were generated by EBV transformation of bone marrow (myeloma patient), peripheral blood, or spleen cells (three SLE patients) as described in detail previously (10). Isotype and idiotype determinations were made by ELISA and have been described previously (10) (Table 1).

RNA Preparation. Total RNA was prepared from 10⁶ cells using guanidinium thiocyanate extraction (10). Determination of variable region gene utilization for both heavy and light chains has been described (10).

Cloning the Productively Rearranged Vκ and Vλ Genes. Cκ primer 5'TGTTCCAGATTTCACCTGCTC3', Cμ primer 5'GAGGGGAAAAGGTGGGGC3', Cy primer 5'GCCAGGGGGGAAGACCATGG3', or Cα primer 5'GGCTAGGGGAGCCAGGAA-CCTTG3' was used to reverse transcribe 10 μg of total RNA into cDNA. The complete PCR has been described in detail elsewhere (10). The primers for the Vκ and Vλ regions were oligonucleotides specific for the 5′ leader sequence or first framework sequences of each appropriate variable region gene family. Vκ1, 5'ACATGGTCCCCGCTCAG3'; or 5'GACATCCAGTTGACCCAG-TC3' (H2F, I-2a); Vλ4, 5'CAGGTCAGCTGCA-CTG3'; Vκ1, 5'GAGGTGTCAGCTGAGAGGCTCAG3', or 5'GACATCCAGTTGACCCAG-TC3' (H2F, I-2a); Vκ3, 5'ATGGAACCCACCGACAGCT3'; Vκ1, 5'CAGGTCACGTGGTGAGCTC3'; (IC4) Vκ3, 5'CAGG-TCGACTGGGGAGGCTC3'; Vκ4, 5'CAGGTCACGTGGTGAGCAGCT3'; and Vκ5, 5'ATGGAACCCACCGACAGCT3'. 30 PCR cycles were performed under the following conditions: denaturation (94°C, 1 min), annealing (48°C, 1 min), and extension (72°C, 3 min). Further amplification of PCR products was performed after elution of DNA from a 2.5% low melt agarose gel (NuSieve GTG; FMC, Rockland, ME). The amplified products were blunt end ligated to either dephosphorylated pGEM or pBluescript (Stratagene, La Jolla, CA) and subcloned into pBluescript (Stratagene, La Jolla, CA). The complete EcoRI digest of the insert was then sequenced as above.

Cloning of Germ Line Vκ1 Genes. Complete EcoRI digests of genomic DNA from bone marrow-derived cells of patient HIC were cloned into the EcoRI site of Charon 16A (13) and >10⁶ phage were screened using a Vκ1-specific DNA coding region probe as described elsewhere (10). Vκ1-positive phages were purified, subcloned into pGEM, and sequenced using the standard Sequenase protocol.

An Mbol partial library from spleen cells of patient DIL was constructed in Charon 40. More than 10⁵ phage were screened using the Vκ1 probe described above. Vκ1-positive phage were purified, subcloned into pBluescript, selected with a CDR1 oligo 5'TTTGATTTCTTTTTTGG3', and sequenced as above.

Myeloma Sera and SLE Sera. Myeloma were obtained from three sources: Dr. Jean-Louis Preud'homme (Poitiers, France), Dr. Peter Wiernick (Albert Einstein College of Medicine, Bronx, NY), and Dr. Alan Solomon (University of Tennessee, Knoxville, TN). SLE serum from one patient was obtained from the clinic at the Bronx Municipal Hospital Center. All were screened for 3I reactivity by ELISA and dsDNA binding by Western blot analysis (14) (Table 2).

IgG from SLE Patient. Purified IgG was obtained from serum by molecular sieve chromatography using a Sephacryl 300 column (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.01 M Tris, 0.001 M EDTA, 0.5 M NaCl, pH 7, as running buffer. DNA binding antibodies were precipitated with dsDNA and then 3I-reactive antibodies were obtained by affinity chromatography (6).

NH2-terminal Amino Acid Sequences of 3I-reactive Light Chains from Myeloma Proteins and SLE Serum. 10 ml of sera from each of six patients with a 3I-reactive myeloma protein was precipitated with 50% ammonium sulphate and the precipitate resuspended and displayed on an analytic IEF gel to determine the isoelectric point of the myeloma protein. The myeloma protein was then purified by preparative IEF. The purity of each protein was determined by SDS-PAGE and analytic IEF gel.

Myeloma proteins and 3I+ serum antibodies were reduced using 6 M urea and either 0.01 M dithiothreitol or 2% mercaptoethanol in 0.1 M Tris, pH 8.0, at 37°C for 2 h and alkylated using 0.05 M iodoacetic acid (15). Heavy and light chains were separated by gel filtration on a Sephacryl 200 column (Pharmacia Fine Chemicals) in 0.05 M Tris, 0.001 M EDTA, 0.1 M NaCl 1% SDS, pH 6.8. The alkylated light chains were subjected to automated sequence analysis (B890M; Beckman Instruments, Fullerton, CA). The initial protein loading was between 5 and 10 nmol and was sequenced in the presence of 2 mg polybrene. The amino acids were identified by two independent reverse phase HPLC methods (16). Protein sequences were then compared with known Ig light chains from the Dayhoff Protein Sequence Bank and were analyzed for the presence of invariant residues of the Vκ families (17).

Results

3I+ Light Chains of EBV-transformed B Cell Lines. The molecular characterization of anti-dsDNA antibodies necessitated that we obtain nucleotide sequences of the light and heavy chain variable regions of the 3I+ antibodies produced by EBV-transformed cell lines. Six of the monoclonal lines and one previously reported, 2A4, (18) express a Vκ1-encoded light chain (Fig. 1). While there is a high degree of homology among the Vκ1 sequences, it is not so striking

| Cell line | Patient | Isotype | DNA binding | Vκ | Vλ |
|-----------|---------|---------|-------------|----|----|
| III-3R    | DIL     | μ       | +           | 1  | 3  |
| III-2R    | DIL     | μ       | +           | 1  | 1  |
| IC4       | HIC     | μ       | +           | 1  | 4  |
| I-2a      | DIL     | γ       | +           | 1  | 3  |
| 1X7RG1    | DIL     | α       | +           | 1  | 3  |
| 2A4       | HIC     | γ       | +           | 1  | 4  |
| R3.5HSG   | RJO     | γ       | +           | 1  | 1  |
| II-1      | DIL     | μ       | +           | 3  | 5  |
| H2F       | HER     | γ       | +           | 4  | 3  |

Characteristics of EBV-transformed B cell lines denoting patient origin, isotype of antibody produced, DNA binding, and Vκ and Vλ family utilization.
as to suggest a common germ line gene. One cell line, II-1, expresses a V\(_{\kappa}\)\_3-encoded light chain (Fig. 2). The remaining line, H2F uses a V\(_{\kappa}\)\_4-derived light chain (Fig. 3). The V\(_{\kappa}J\) joins of each of the five rearranged lines from patient DIL and of the two lines from patient HIC differ, indicating that the B cells within each set are not clonally related. The deduced amino acid sequences of the V\(_{\kappa}\)\_1 light chains reveal conserved framework (FW) regions (Fig. 4).
be the template for our 3I + antibodies, we screened the VK1 library with a VK1 probe and isolated >30 clones expressing antibodies are derived.

This is especially true for FW2. Interestingly, the FWs of the V_K3- and the V_K4-encoded light chains are also highly homologous to the V_K1 protein sequences, providing a potential explanation for their 3I reactivity. The partial protein sequences of the 3I+ myeloma proteins are remarkably similar to the deduced amino acid sequences of the 3I+ antibodies (Fig. 5). Similarly, the consensus sequence of the light chains of 3I+ serum anti-dsDNA antibodies from the lupus patient, SNA, exhibits very strong homology to the light chains of the myeloma proteins and to the predicted amino acid sequences of the light chains of the EBV-transformed lines (Fig. 5). The analysis of these primary amino acid sequences reveals many regions of homology, yet no unique region that might represent the 3I idiotope.

All the antibodies except R3.5H5G bind dsDNA. Many DNA binding antibodies and other DNA binding proteins are enriched for arginine, asparagine, and glutamine residues. In anti-DNA antibodies, these residues are present in CDRs and presumably are contact residues for antigen (19-23). Here we find an unusually high number of these residues in CDR1. A random sampling of V_K1 light chains shows that on average 2.8 such residues are present in CDR1 (17). The light chains of the eight dsDNA binding antibodies reported here contain an average of 3.75 (range, 3-5). The R3.5H5G antibody is the only antibody that does not exhibit any specificity for dsDNA and it possesses only two such residues in CDR1. Although most of the myeloma proteins also bind dsDNA (see Table 2), the incidence of arginine, glutamine, and asparagine residues in the myeloma proteins is not so striking as in the antibodies from the EBV-transformed B cell lines.

Comparison of Expressed 3I Sequences to Their Putative Germine Genes. The human V_K1 gene family is the largest of the four kappa gene families, containing ~30 genes (24-26). Our previous study of the 2A4 cell line revealed ~45 nucleotide differences between the most homologous V_K1 germ line gene and the 2A4 light chain nucleotide sequence, suggesting that no published V_K1 gene was the germ line gene template for 2A4 (18). This observation prompted us to construct genomic libraries from both the lupus patient DIL and myeloma patient HIC with the hope of obtaining new V_K1 genes that might be the germ line genes from which our expressed antibodies are derived.

We screened an HIC bone marrow library and a DIL spleen library with a V_K1 probe and isolated >30 clones hybridizing to a V_K1 fragment (10). To identify those V_K1 genes that had not been previously reported and that might be the template for our 3I+ antibodies, we screened the V_K1 clones with an oligonucleotide probe highly homologous to the CDR1 sequence of two of the three IgM antibodies. This CDR sequence was not present in any previously reported V_K1 gene and so was likely to identify new V_K1 genes in our libraries. Using this probe, we isolated from the HIC library two new functional germ line genes and one pseudogene, as well as two new pseudogenes from the DIL library. The germ line gene DILpl1 is the only one of the genes we cloned with strong homology to any of the 3I+ sequences reported here (Fig. 6). This V_K1 gene is quite unusual; codon 88, GGT, encodes glycine, whereas all V_K1 genes reported to date encode cysteine at this codon. As shown in Fig. 1, all the expressed 3I+ light chain variable regions encode cysteine at codon 88 (TGT).

We then compared the light chain nucleotide sequences of the V_K1 3I+ antibodies to the DIL-pl gene or to the most homologous germ line sequence available in the literature (Table 3). The III-2R IgM line derived from DIL is the most homologous to DIL-pl, differing by 17 nucleotides. Two others, I-2a and 2A4, are more homologous to DIL-pl than to any other reported V_K1 gene but differ by 32 and 37 nucleotides, respectively (Table 3). Three V_K1 cell lines, III-3R, IC4, and IX7RG1, show most homology to the germ line gene HK137 (Table 3). In fact, the III-2R line is also more homologous to HK137, differing by 15 nucleotides, than to DIL-pl; however, it displays more homology in FW regions to DIL-pl than to HK 137 (25, 27). The V_K1 sequence from the non-DNA binding R3.5H5G cell line is most homologous to the germ line gene b (Table 3).

Calculation of amino acid replacement to silent changes (R/S) (Table 3) indicates that almost all the V_K1 antibodies bind dsDNA.
Table 3. Comparison of VK Sequences to Most Homologous Germ Line Gene

| Germ line gene | Cell line   | Percent homology | R/S ratios | R/S per domain |
|----------------|-------------|------------------|------------|----------------|
|                |             |                  | FWs        |                |
|                |             |                  | Total R/S  |                |
| DILp1          | III-2R(μ)   | 96               | 8 5 10     | 1:0 1:0 1:5    |
|                | 2A4(γ)      | 87               | 25 13 12   | 5:2 2:0 6:10   |
|                | I-2a(γ)     | 89               | 19 8 11    | 1:0 3:2 4:9    |
| HK137          | III-3R(μ)   | 91               | 13 5 8 10  | 0:1 3:6 3:2 4:1|
|                | IC4(μ)      | 92               | 10 5 5     | 0:3 3:2 3:0 4:0|
|                | III-2R(μ)   | 95               | 10 4 5 3 3 | 0:2 3:0 1:4 0:1|
|                | 1X7RG1(αε) | 95               | 9 5 4     | 2:2 1:2 2:0 1:2|
| b'             | R3.5H5G(γ) | 92               | 11 3 8 3 1 | 1:1 2:2 0:5 4:1|
| VK328          | II-1(μ)     | 98               | 3 2 1 2 0 | 0:0 0:0 2:1 1:0 |
| VK4           | H2F(γ)      | 98               | 3 3 0 3 1 2 | 3:0 0:0 0:0 1:2 0:0 |

Replacement (R) to silent (S) ratios in the light chains of the 31+ antibodies. The ratio of mutations that result in an amino acid replacement to those that are silent are tabulated for the FWs and the CDRs.
92 in CDR3 when compared to the sequence encoded by the b' germ line gene (Table 6).

Pathogenic anti-DNA antibodies are thought to be cationic (31). Among the V,1 light chains, there is no trend to acquire a more positive net charge. Although cationic anti-DNA antibodies are preferentially sequestered in the kidney, the V,1 light chains, there is no trend to acquire a more positive net charge. Although cationic anti-DNA antibodies are preferentially sequestered in the kidney, most of the V,1 sequences expressed in our panel of anti-DNA B cells are more negatively charged than the deduced amino acid sequence of the germ line genes.

Two of the 31+ sequences are not encoded by V,1 genes. The II-1 light chain derives from a V,3 gene. It differs from the presumed germ line-encoded amino acid sequence by two amino acids in FW regions and two in CDRs (Table 7). The expressed antibody reflects the acquisition of an asparagine in CDR1 and the loss of a glutamine in CDR3. Similarly, the V,4-encoded H2F light chain differs by only three amino acids from the putative germ line template (Table 8). The single substitution in a CDR replaces an alanine with a threonine. The amino acid substitutions in II-1 and H2F are too few to argue for or against antigen selection.

31+ Antibodies Are Encoded by Four V, Gene Families. The V, gene segments encoding the expressed heavy chains of the 31+ antibodies are derived from four families: V,1, V,3, V,4, and V,5. The nucleotide sequences, including that of 2A4 (18), are shown in Fig. 7. The predicted protein sequences of the heavy chains are presented in Fig. 8. If the appropriate germ line genes have all been cloned, our sequence data suggest that the heavy chains are also somatic mutants of their germ line counterparts. Table 9 presents the replacement to silent substitution ratios in FW and CDR regions of each antibody compared to the sequence encoded by the most homologous published V, germ line gene. In general,
similar to what was seen in the light chains, there is a lower than random R/S ratio in FW regions and a higher than random P/S ratio in CDRs. When the actual amino acid substitutions are examined, a net accumulation of asparagine, arginine, or glutamine is seen in five antibodies and a net loss in four (Tables 10-13). Similarly, four anti-DNA antibodies show a net gain in positive charge, and three show a net loss. One anti-DNA antibody shows no change in charge and R3.5H5G, which does not bind DNA, also shows no change in charge.

**CDR3 Regions Differ among Anti-DNA Antibodies.** Fig. 9 shows that a variety of D segments are used by these EBV lines. In fact, three lines, III-3R, III-2R, and 2A4, express D-D fusions, a previously reported feature of autoantibodies (18, 32). Other lines express D regions that have yet to be reported and differ from each other as well as from the reported D region genes.

J usage is also not restricted among 31+ or DNA binding EBV lines, although J4 is used in seven of the nine lines (Fig. 9). All J4+ antibodies have a single silent nucleotide change from the published J4 sequence (CAG instead of CAA) (33). This change may represent a polymorphism within the population similar to what others have reported for J6 (34). As in the light chain sequences, there is no common junction evident among antibodies deriving from a single individual. We, therefore, cannot establish any genealogy by examining the heavy chain genes.

**Discussion**

Structural analyses of antibodies provide information about the basis for both idiotype expression and antigenic specificity. Molecular genetic studies can provide insights about the B

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**Table 4. Comparison of 31\* V\*1 Antibodies to DILp1**

| Amino acid | DILp1 | III-2R | 2A4 | I-2A |
|------------|-------|--------|-----|------|
| FW1        |       |        |     |      |
| 2          | 1     | -      | L   | -    |
| 3          | Q     | -      | K   | -    |
| 4          | L     | M      | M   | -    |
| 10         | S     | -      | -   | F    |
| 12         | S     | -      | P   | -    |
| 18         | R     | -      | T   | -    |
| CDR1       |       |        |     |      |
| 25         | V     | A      | A   | A    |
| 28         | G     | -      | S   | D    |
| 30         | S     | -      | N   | R    |
| 31         | S     | N      | R   | N    |
| 32         | Y     | -      | F   | -    |
| 34         | N     | A      | -   | T    |
| FW2        |       |        |     |      |
| 37         | R     | Q      | Q   | Q    |
| 43         | V     | -      | A   | A    |
| 46         | L     | -      | -   | V    |
| CDR2       |       |        |     |      |
| 50         | S     | A      | G   | P    |
| 53         | N     | T      | S   | T    |
| FW3        |       |        |     |      |
| 58         | V     | -      | F   | -    |
| 59         | P     | -      | S   | -    |
| 70         | D     | -      | E   | E    |
| 76         | S     | -      | R   | I    |
| 83         | V     | -      | F   | F    |
| 88         | G     | C      | C   | C    |
| CDR3       |       |        |     |      |
| 90         | R     | K      | Q   | Q    |
| 91         | T     | Y      | S   | L    |
| 92         | Y     | N      | -   | I    |
| 93         | N     | S      | S   | S    |
| 94         | A     | -      | T   | Y    |

**Table 5. Comparison of 31\* V\*1 Antibodies to HK137**

| Amino acid | Hk137 | III-3R | III-2R | 1X7RG1 | IC4 |
|------------|-------|--------|--------|--------|-----|
| FW1        |       |        |        |        |     |
| 4          | M     | -      | -      | I      | -   |
| 6          | Q     | -      | -      | H      | -   |
| CDR1       |       |        |        |        |     |
| 24         | R     | Q      | -      | -      | Q   |
| 25         | A     | -      | -      | T      | -   |
| 28         | G     | D      | -      | -      | D   |
| 31         | N     | -      | -      | K      | -   |
| 34         | A     | N      | -      | -      | N   |
| FW2        |       |        |        |        |     |
| 36         | F     | Y      | Y      | -      | Y   |
| 43         | A     | -      | V      | -      | -   |
| 46         | S     | L      | L      | -      | L   |
| 48         | I     | -      | -      | L      | -   |
| CDR2       |       |        |        |        |     |
| 50         | A     | D      | -      | N      | D   |
| 51         | A     | -      | -      | P      | -   |
| 53         | S     | N      | T      | -      | N   |
| 55         | Q     | E      | -      | -      | E   |
| 56         | S     | T      | -      | -      | R   |
| FW3        |       |        |        |        |     |
| 62         | F     | I      | -      | -      | -   |
| 65         | S     | -      | -      | -      | G   |
| 73         | L     | F      | -      | -      | F   |
| 76         | S     | -      | -      | N      | -   |
| 77         | S     | -      | -      | N      | -   |
| 83         | F     | I      | V      | -      | I   |
| CDR3       |       |        |        |        |     |
| 90         | Q     | -      | K      | -      | -   |
| 92         | N     | D      | H      | D      | -   |
| 93         | S     | N      | -      | -      | T   |
| 94         | Y     | L      | A      | -      | L   |

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We have determined the light and heavy chain variable region gene sequences of nine EBV-transformed 3I+ clonal B cell lines. All except one antibody binds to dsDNA. Because elevated titers of 3I+ antibodies are associated in SLE patients with glomerulonephritis and since we have documented that 3I+ antibodies are present in the kidneys of patients with SLE, the antibodies that bind to dsDNA are presumed to include representatives of a pathogenic subset present in a large percent of patients with SLE.

Previously, we addressed questions regarding general molecular characteristics of EBV-transformed 3I+ B cell lines (10). We found that 3I+ antibodies were encoded mainly by VK1 light chain genes regardless of patient origin. We believe EBV transformation does not introduce experimental bias in creating this VK restriction for two reasons. First, these lines express light chains with amino acid sequences that are homologous to the sequence of 3I+ polyclonal serum antibodies from a SLE patient and to a number of 3I+ myeloma proteins. Second, other reports of VK gene usage in EBV-transformed B cells show a variety of expressed VK genes (34, 35). It is likely, therefore, that the data reflect a disease-associated, as well as idiotype-associated, V region restriction as has been proposed by Shen et al. (36) in chronic lymphocytic leukemia.

We wished to determine if sequence analysis could reveal residues or regions among the VK1, VK3, and VK4 antibodies that constitute the 3I epitope or that participate in DNA binding. Our results indicate that the 3I+ VK1 antibodies are closely related on both the nucleotide and the protein level. In fact, the data show strong conservation of sequence in FW regions among all the antibodies. This conservation is evident in the partial protein sequences of the 3I+ myeloma light chains as well. The greater homology in FWs than in CDRs supports the hypothesis that the 3I determinant(s) lies within the framework regions of the light chain. In general, it has been difficult to map antibody idiotypes; in the 3I system also, more definitive analysis using peptide scanning and site-directed mutagenesis will be required to localize the idiotype.

Sequence analysis of anti-DNA antibodies from various mouse strains has led to the hypothesis that the presence of particular amino acids in the CDRs is associated with the ability to bind to DNA. A number of amino acids, including asparagine, glutamine, and arginine, have been postulated to interact with the major and minor grooves of dsDNA (19). These have been implicated in the antigenic specificity of anti-DNA antibodies. Amino acid sequence analysis of mouse anti-DNA antibodies has led to the interesting observation that there is an increase in the number of arginines and tyrosines over that of previously sequenced mouse antibodies (20, 21, 37). It has also been suggested that DNA binding may select for arginine in position 98 of mouse heavy chain variable regions (38). In the heavy chain variable region of human anti-DNA antibodies, tyrosine residues at position 32 and/or 33 as well as Ser-Thr-Asn-Tyr-Asn in CDR2 have been postulated to form the DNA binding pocket in anti-DNA antibodies (37).

Our data show that the number of arginine, glutamine,
asparagine, and tyrosine residues in CDR1 of the κ chain variable region is distributed as follows: in the eight DNA binding antibodies, four contain five putative DNA-interacting amino acids and four antibodies contain four such residues, while R3.5H5G, the one antibody that shows no dsDNA binding, contains only two such residues (Fig. 4). Additionally, the total number of asparagine residues in CDRs of the entire light chain variable region of the DNA binding antibodies is higher than in the one non-dsDNA binding antibody. Four antibodies contain four asparagine residues, three contain two, and one contains one. R3.5H5G, which does not bind dsDNA, encodes no asparagine residues. Examination of a random population of previously sequenced human Vκ1, Vκ3, and Vκ4 antibodies reveals that they possess fewer asparagine residues than the 3I+ DNA binding antibodies we report here (mean, 1.33; n, 12) (17).

While it has been suggested that tyrosine residues in CDRs of the heavy chain in mouse may be significant in DNA binding, we do not find any significant increase in tyrosine residues in our set of anti-DNA antibodies. In fact, R3.5H5G, the one 3I+ antibody that does not bind dsDNA, contains two juxtaposed tyrosine residues within CDR1 of the heavy chain.

In searching the sequences of our antibodies for ATP binding site motifs, Gly-X-Gly-Lys-Thr, Gly-(X)4-Gly-Lys-Thr-(X)6-Ile/Val, or Gly-X-Gly-X-Gly/Ser, we find stretches of Gly residues interspersed with Ser residues within FW regions. These regions appear, however, to be conserved among all the Vκ families and not to be specific for V regions used to form anti-DNA antibodies.

While the three-dimensional structure of a folded polypeptide chain is determined by its primary sequence, similar

| Germ line gene | Cell line | Percent homology | Total FWs | CDRs | R/S per domain |
|---------------|-----------|------------------|-----------|------|----------------|
| 51P1*         | III-2R(μ) | 96               | 3 1 2     | 8 5 3 | 0.2 0.0 1.0 1.1 | 4:2 |
| H16BR1        | III-3R(μ) | 90               | 18 7 11   | 7 5 2 | 1.4 2.1 4.6 3.0 | 2:2 |
| 71-4S         | IC4(μ)    | 97               | 10 7 3    | 3 3 0 | 3.0 1.3 3.0 1.0 | 2:0 |
| 56P1*         | I-2a(γ)   | 94               | 3 3 0     | 9 7 2 | 0.0 0.0 3.0 2.1 | 5:1 |
| Vκ265         | 1XT7RG1(α)| 89               | 18 8 10   | 12 10 2 | 4.2 0.3 4.5 3.0 | 7:2 |
| 71-2S         | 2A4(γ)    | 92               | 15 5 10   | 7 7 0 | 2.3 2.3 1.4 1.0 | 6:0 |
| 206P3*        | R3.5H5G(γ)| 94               | 12 6 6    | 9 7 2 | 3.0 1.2 2.4 2.1 | 5:1 |
| 251S          | II-1(μ)   | 98               | 4 2 2     | 5 5 0 | 1:1 0:1 1:0 2:0 | 3:0 |
| Vκ265         | H2F(γ)    | 90               | 16 6 10   | 11 9 2 | 2.4 1:1 3:5 2:1 | 7:1 |

Replacement (R) to silent (S) ratios in the heavy chains of the 3I+ antibodies. The ratios of mutations that result in an amino acid replacement to those that are silent are tabulated for the FWs and the CDRs.

* Rearranged gene of fetal origin.
† Germ line pseudogene.
§ Closest homologous germine gene.

Table 9. Comparison of Vκ Sequences to Most Homologous Germline Genes

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Figure 8. Derived heavy chain amino acid sequences of EBV-transformed cell lines. 2A4 has a two-amino acid insertion in CDR2. *NY.
folds can be formed by very different sequences. It is likely that there are multiple ways different amino acids combine to form an antigen binding pocket with affinity for dsDNA antigenic epitopes. Different idiotypic systems may display different motifs, and even within an idiotypic system different motifs may be present. The specificity of a particular amino acid will depend on the conformation and orientation of the protein backbone. Such observations may help explain the apparent acquisition of dsDNA binding in the V \textsubscript{K1}-encoded antibodies as a result of presumed somatic mutation despite no actual increase in the number of amino acids capable of contacting DNA. It is also important, however, to consider that DNA need not be the eliciting antigen and some amino acid substitution may be present to provide increased binding to an unidentified antigen.

Based on the S107-U4 paradigm in which an antibacterial antibody, S107, underwent a single amino acid substitution in CDR1 and acquired binding to dsDNA, we have hypothesized that somatic mutation in vivo may also lead to DNA binding (39). In fact, all anti-dsDNA antibodies derived from individuals with SLE or lupus-prone mice that have been sequenced so far appear to show somatic mutations (21, 22, 40, 41). An analysis of the somatic mutations present in the expressed V region sequences can help determine if the antibody response is antigen driven or is a consequence of polyclonal activation, a major question in the study of the anti-DNA response. If somatic mutations occur randomly throughout the V region yielding a replacement to silent mutation ratio of 2.9:1, then the antibodies may reflect B cell or T cell polyclonal activation. If, however, mutations are clustered in CDRs or if the R/S ratio in CDRs is >2.9:1, then antigen selection may play a role in expanding particular B cells clones. The somatic mutations seen in some anti-dsDNA antibodies previously appear to lead to the acquisition of DNA binding, and the unmutated germ line genes may be presumed to encode antibodies with little or no affinity for dsDNA. For example, the sequence of the 2A4 anti-dsDNA antibody displays substitutions that lead to a more cationic antibody and one containing more putative DNA binding residues in CDRs (18).

We have performed an analysis of the presumed somatic mutations present in the 3I+ light chains sequences reported here. Because all the human \textit{V}_{\textsubscript{K}} genes have not yet been cloned and sequenced, we attempted to isolate additional \textit{V}_{\textsubscript{K1}} germline genes. Of the five we sequenced, two were

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### Table 10. Replacement Mutations in 3I+ \textit{V}_{\textsubscript{a1}} Sequences

| Amino acid | 51P1 | III-2R |
|------------|------|--------|
| CDR1  | 33   | A      | T   |
| CDR2  | 50   | G      | R   |
|       | 52   | I      | M   |
|       | 55   | F      | L   |
|       | 57   | T      | L   |
| FW3   | 74   | E      | K   |

### Table 11. Replacement Mutations in 3I+ \textit{V}_{\textsubscript{a3}} Sequences

| Amino acid | VH26 | H2F | 1X7RG1 |
|------------|------|-----|--------|
| FW1  | 1    | E   | Q     |
|       | 5    | L   | V     |
|       | 13   | Q   | K     |
|       | 19   | R   | T     |
|       | 28   | T   | I     |
| CDR1  | 31   | S   | D     |
|       | 33   | A   | Y     |
|       | 35   | S   | N     |
| FW2   | 37   | V   | I     |
| CDR2  | 50   | A   | Y     |
|       | 51   | I   | T     |
|       | 53   | G   | S     |
|       | 54   | S   | R     |
|       | 56   | G   | S     |
|       | 57   | S   | T     |
| FW3   | 75   | S   | A     |
|       | 78   | T   | S     |
|       | 95   | Y   | F     |
|       | 98   | K   | R     |

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pseudogenes. This finding is consistent with the reported high frequency of pseudogenes among human V region genes. We believe that all the 3I⁺ light chains we have described have undergone somatic mutation. Studies on selected lines have shown the absence of CDR nucleotide sequences in the patient's germline DNA, confirming the somatic acquisition of the CDR nucleotide sequences. It is clear, however, that the description of human V region genes is far from complete and that there is substantial allelic polymorphism in the population; therefore, the mutational analysis remains speculative. When 3I⁺ antibody genes are compared to the most homologous germline gene sequences available, they exhibit between 89% and 96% homology to the closest Vζ germ line gene. Most of the Vζ-encoded 3I⁺ antibodies display high R/S ratios in CDRs, suggesting substantial changes in antigen binding from their germ line-encoded counterparts. The analysis of these antibodies suggests that antigen selection drives the anti-DNA response, and it is possible that the parental B cell may be initially triggered by an antigen only distantly related to DNA. II-1 exhibits 98% homology to the single reported Vκ4 germ line gene. For these two antibodies, the number of mutations is too small to speculate on whether they represent antigen selection or not.

Our previous studies revealed that the Vζ gene utilization among this panel of EBV cell lines was quite heterogeneous (10). Among the antibodies described here, four of the six Vζ families are represented: Vζ1, Vζ3, Vζ4, and Vζ5. The Vζ1 and Vζ3 families are the largest families, including >30 genes in each, whereas Vζ4 and Vζ5 are significantly smaller (6–10 genes and 2–3 genes, respectively) (42). The distribution of Vζ gene usage by our panel of antibodies probably reflects on the size of each family and shows no restriction.

The D regions expressed by this group of lines are quite unusual. Only one of the eight lines newly reported, III-3R, expresses an already identified D gene. III-3R, III-2R, and

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**Table 11.** Replacement Mutations in 3I⁺ Vζ4 Sequences

| Amino acid | 56P1 | I-2a |
|------------|------|------|
| CDR1       |      |      |
| 32         | Y    | S    |
| 33         | A    | P    |
| CDR2       |      |      |
| 50         |      |      |
| 53         | V    | F    |
| 54         | Y    | L    |
| 57         | D    | E    |
| 58         | N    | S    |
| FW3        |      |      |
| 68         | F    | L    |
| 84         | N    | S    |
| 98         | R    | S    |

| Amino acid | H16BR | III-3R |
|------------|-------|--------|
| FW1        | 9     | stop   |
| 32         | S     | Y      |
| 33         | W     | R      |
| 35         | H     | S      |
| FW2        | 38    | C      |
| 42         | E     | G      |
| CDR2       | 50    | D      |
| 53         | C     | Q      |
| FW3        | 83    | V      |
| 91         | M     | T      |
| 92         | T     | A      |
| 97         | V     | A      |

**Table 12.** Replacement Mutations in 3I⁺ Vζ4 Sequences

| Amino acid | 71-4 | IC4 |
|------------|------|-----|
| FW1        | 6    | Q   |
| 29         | S    | I   |
| 30         | G    | S   |
| CDR1       | 32   | Y   |
| FW2        | 40   | P   |
| 54         | S    | T   |
| CDR2       | 50   | Y   |
| 54         | D    |
| 55         | Y    |
| 58         | S    |
| 59         | T    |
| 60         | N    |
| FW3        | 73   | V   |

| Amino acid | V71-2 | 2A4* |
|------------|-------|------|
| FW1        |       |      |
| 16         |       |
| 29         | E     |
| 33         | S     |
| FW2        | 43    | P    |
| 49         | W    |
| CDR2       | 52    | Y    |
| 54         | Y    |
| 55         | Y    |
| 58         | S    |
| 59         | T    |
| 60         | N    |
| FW3        | 73    | V    |

* Previously published data.
Table 13. Replacement Mutations in 3l+ V_n5 Sequences

| Amino acid | 251 | II-1 |
|------------|-----|------|
| FW1        | 30  | T    |
| CDR1       | 31  | S    |
| CDR2       | 50  | I    |
| FW3        | 75  | S    |

2A4 express D-D fusions, a previously reported feature of autoantibodies (18, 32). The remaining six antibodies express D segments of unknown origin. It is possible that these D segments are highly diversified through somatic mechanisms or, alternatively, derive from germline Ds not yet described. J_n usage is possibly restricted as seven of nine lines utilize J_n4.

Most of the expressed V_n genes are 89-98% homologous to previously published functional germ line genes, germ line pseudogenes, or rearranged genes derived from human fetal tissue (presumably germ line in sequence). It is apparent that the data presented in Table 9, showing replacement to silent substitution ratios for both FWs and CDRs, indicate that, similar to what is seen in the light chains, the heavy chains show strong conservation in FW sequences and high CDR substitution rates. However, unlike the light chains, there appear to be few arginine, asparagine, and glutamine replacements, suggesting perhaps that DNA binding by these EBV lines is more a function of the light chain than the heavy chain.

Van Es et al. (43) described an IgG anti-DNA antibody from an EBV-transformed cell line derived from an SLE patient encoded by V_KIIIb and V_n4 genes. Similar to the antibodies we report, this antibody appears to display more replacement mutations in CDRs than in FW regions. Also, like the antibodies we report, only one out of six presumed replacement mutations in the heavy chain CDRs enriches for a potential DNA binding amino acid, while the light chain replacement mutations enrich for arginine and asparagine.

Our earlier studies on myeloma proteins showed that 3l+ IgM antibodies are non-DNA binding, while the most 3l+ IgG proteins bind to dsDNA (14). This observation on the association of DNA binding with the IgG isotype is in contrast to studies on the EBV lines that we report here, where both IgM and IgG antibodies bind DNA although with varying affinities (10). While IgM myeloma proteins are often encoded by unmutated germ line genes, the IgM antibodies produced by our EBV lines are most probably mutated. This may be due to antigen selection for mutated IgMs or perhaps to a mechanism of hypermutation in SLE.

These studies demonstrate some of the difficulties in structural and genetic studies of antibody responses. While our initial study of a single anti-dsDNA antibody suggested antigen selection and amino acid substitutions leading to an increased affinity for DNA, the analysis of the additional seven DNA binding antibodies reported here suggests that selection for a more cationic antibody or for DNA binding residues in CDRs is not an obvious feature of all or even most of the antibodies. It is probable, however, that multiple forces act on the anti-DNA response and that the selection of the expressed repertoire is determined by forces such as idiotype as well as antigen, DNA, or otherwise. The analysis of a large number of human anti-DNA antibodies, including multiple antibodies from individual patients, shows that there is no single paradigm for explaining structural or molecular genetic features of these autoantibodies. The structural basis of the 3l idiotype cannot be localized from our amino acid sequences. Idiotypic determinants may well be conformation dependent and the accumulation of sequence data alone may be insufficient for idiotype localization. The CDR1 domain of the light chain may well play a critical role in the binding of these antibodies to dsDNA, although this motif alone may not be sufficient. Site-directed mutagenesis of these antibody genes will be necessary to confirm this hypothesis. The analysis of presumed somatic mutations in these antibodies is complex. Human V region genes have not been analyzed extensively enough to make mutational analysis more than suggestive. Nevertheless, the preliminary analysis of these antibodies shows that mutations do not always lead to an increase in dsDNA binding residues or in antibody charge. More information on anti-DNA sequences and the human V region germ line repertoire, including the extent of allelic polymorphism, is necessary in order to understand fully the origins of an autoantibody response and to elucidate the forces that determine the expressed antibody repertoire in SLE.

Figure 9. Junctional nucleotide sequences of heavy chains. Several D regions could not be identified in the literature and are indicated as unknown (UNK).
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