Lupus-specific antibodies reveal an altered pattern of somatic mutation.

A J Manheimer-Lory, … , C Aranow, B Diamond

Research Article

The F4 idiotype is a heavy chain determinant expressed almost exclusively on IgG immunoglobulins and is highly associated with specificity for double-stranded DNA. Since high-titered F4 expression is present predominantly in sera of patients with systemic lupus erythematosus (SLE), we thought F4+ IgG antibodies might constitute a useful subset of immunoglobulins in which to investigate lupus-specific alterations in variable (V) region gene expression or in the process of somatic mutation. This molecular analysis of F4+ B cell lines generated from lupus patients demonstrates that despite the strong association of F4 reactivity with specificity for native DNA, there is no apparent VH gene restriction. Furthermore, VH gene segments encoding these antibodies are also used in protective immune responses. An examination of the process of somatic mutation in F4+ antibodies showed no abnormality in frequency of somatic mutation nor in the distribution of mutations in complementarity-determining regions or framework regions. However, there was a decrease in targeting of mutations to putative mutational hot spots. This subtle difference in mutations present in these antibodies may reflect an intrinsic defect in mutational machinery or, more likely, altered state of B cell activation that affects the mutational process and perhaps also negative selection.

Find the latest version:

Lupus-specific Antibodies Reveal an Altered Pattern of Somatic Mutation

Audrey J. Manheimer-Lory,* Gisele Zandman-Goddard,* Anne Davidson,*‡ Cynthia Aranow,§ and Betty Diamond*‡

*Department of Microbiology and Immunology, and ‡Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461; and §Department of Medicine, State University of New York at Downstate, Brooklyn, New York 11225

Abstract

The F4 idiotype is a heavy chain determinant expressed almost exclusively on IgG immunoglobulins and is highly associated with specificity for double-stranded DNA. Since high-titered F4 expression is present predominantly in sera of patients with systemic lupus erythematosus (SLE), we thought F4$^+$ IgG antibodies might constitute a useful subset of immunoglobulins in which to investigate lupus-specific alterations in variable (V) region gene expression or in the process of somatic mutation. This molecular analysis of F4$^+$ B cell lines generated from lupus patients demonstrates that despite the strong association of F4 reactivity with specificity for native DNA, there is no apparent V_H gene restriction. Furthermore, V_H gene segments encoding these antibodies are also used in protective immune responses.

An examination of the process of somatic mutation in F4$^+$ antibodies showed no abnormality in frequency of somatic mutation nor in the distribution of mutations in complementarity-determining regions or framework regions. However, there was a decrease in targeting of mutations to putative mutational hot spots. This subtle difference in mutations present in these antibodies may reflect an intrinsic defect in mutational machinery or, more likely, altered state of B cell activation that affects the mutational process and perhaps also negative selection. (*J. Clin. Invest.* 1997. 100:2538–2546.) Key words: autoreactivity • anti–double-stranded DNA antibody • idiotype • mutational hot spots • variable region gene expression

Introduction

Idiotypic analyses have been useful in revealing the molecular genetics of specific antibody responses. In particular, idiotypic analyses of anti-DNA antibodies have helped reveal the structural and genetic heterogeneity that characterizes this pathogenic autospecificity. Data from several laboratories demonstrate that most idiotypic specificities present on anti-DNA antibodies are also present on normal serum Igs (1). This observation implies the existence of a structural and genetic relationship between autoantibodies and antibodies that are pre-sumably part of a protective immune response. However, a few idiotypes are highly restricted to DNA-binding antibodies and to individuals with SLE (2, 3). Analysis of the molecular genetics of these idiotypes may provide clues to SLE-specific Ig expression.

We have identified three lupus-associated anti-DNA idiotypes. 3I and 8.12 are expressed in high titer in lupus sera, yet are also present on antipneumococcal antibodies from nonautoimmune individuals (4, 5). The 3I idiotype is present on κ light chains, primarily those encoded by variable (V)^l region κI genes (6), while the 8.12 idiotype is encoded exclusively by VλII light chain genes (7). Both idiotypic determinants can be encoded by germ line genes without somatic mutation, although all anti-DNA antibodies expressing these idiotypes display evidence of somatic mutation. We have interpreted these observations to mean that 3I$^+$ and 8.12$^+$ antibodies can be generated routinely in response to bacterial antigens; specificity for DNA in these idiotypic systems may be generated somatically, and a defect in the regulation of autoreactive B cells in individuals with SLE may permit the survival of cells acquiring anti-DNA specificity.

The F4 idiotype is an anti-DNA–associated idiotype that is not present in the repertoire of nonautoimmune individuals or on antipneumococcal antibodies. It is present in high titer in ∼ 50% of lupus patients with anti–double-stranded (ds) DNA activity, and from 30 to 50% of anti-DNA antibodies are F4 reactive (3). The F4 determinant is present on the heavy chain variable region, and is expressed almost exclusively on IgG antibodies (3). These observations have led us to suggest that both the F4 idiotype and DNA binding are acquired by somatic mutation, and that the F4 idiotype is intimately related to DNA specificity. As high-titered expression of this idiotype is present in SLE, a molecular genetic characterization of F4$^+$ antibodies might reveal differences in Ig variable region gene expression or in the process of somatic mutation in lupus patients that might predispose them to autoantibody production.

In this study, we characterize 10 F4$^+$ mAbs, 8 of which are novel, and 2 of which have been reported previously (6). The molecular genetic analysis of these antibodies confirms previous serologic studies, demonstrating a strong association of the F4 idiotype with IgG isotype and with DNA binding. F4 heavy chains are encoded by V_H genes that are used in protective antibody responses. A comparison of somatic mutation in these antibodies and in antibodies from nonautoimmune individuals encoded by the same V_H genes shows a subtle alteration in the pattern of mutation in SLE antibodies. Somatic mutation in the SLE V_H gene is targeted to a lesser extent to known mutational hot spots, suggesting an intrinsic B cell defect or an alteration in B cell activation in SLE.

1. *Abbreviations used in this paper:* CDR, complementarity-determining region; ds, double-stranded; FR, framework region; R, replacement; S, silent; Sm, Smith antigen; V, variable (region); V_H, heavy chain variable region; V_L, light chain variable region.

Table I. F4+ EBV-transformed Cell Lines

Patient	Line	Isotype	3I	V_H	J_H	V_L	J_L
A*	78	γ, κ	+	3	5	κ2	2
B‡	5–3	γ, κ	+	3	4	κ3	1
B‡	12–2	γ, κ	+	3	6	κ2	4
B‡	17–3	γ, λ	−	3	4	λ3	λ2/3
H§	17	γ, λ	−	3	4	λ3	λ2/3
M*	36	γ, κ	+	1	3	κ1	2
MC‖	90	γ, κ	+	3	4	κ1	2
O*	50	γ, λ	−	4	3	λ3	λ2/3
D¶	I–2a	γ, κ	+	3	4	κ1	4
H*‡	H2F	γ, κ	+	3	4	κ4	1

Characteristics of F4+ EBV lines denoting patient origin, isotype, 3I reactivity, and $V_H J_H$ and $V_L J_L$ usage. Patient clinical status at time of blood drawing: *renal disease; ‡arthritis; §sample was obtained 4 yr later than H2F, when patient had only arthritis; ‖asymptomatic; ¶thrombocytopenia.

Table III. Comparison of Rearranged V Regions with Germ Line Genes

Clone	V_H	Germ line*	NA‡	AA§	V_L	Germ line*	NA‡	AA§
A78	3	DP35	93.9	87.8	κ2	A2	87.6	83.2
B5–3	3	DP49	94.2	88.8	κ3	A27	96.5	92.6
B12–2	3	DP51	90.1	86.7	κ2	A2	86.3	83.2
B17–3‖	3	DP47	94.5	86.6	λ3	IGLV3S2	96.2	96.5
H17‖	3	DP47	95.9	90.8	λ3	III.1	95.3	94.2
M36	1	hv1263	89.5	82.7	κ1	08	96.4	90.5
MC90	3	DP58	96.3	94.9	κ1	08	98.0	96.8
050‖	4	71–4	96.2	89.7	λ3	hlv318	96.9	96.5
I–2a¶	3	hv3005–f3	93.4	90.8	κ1	L8	95.0	91.6
H2F¶	3	DP47	89.8	80.6	κ4	B3	98.0	93.1

Summary of germ line gene usage by F4+ EBV-transformed lines. *References 10–13. ‡Percentage of nucleic acid (*NA*) homology. §Percentage of amino acid (*AA*) homology. ‖Homologies for these lines only reflect sequence beginning at codon 10. ¶Reference 4.

Methods

Generation of F4+ B cell clones

Peripheral blood from six individuals (designated A, B, H, M, MC, and O) with SLE (as defined by the American College of Rheumatology criteria) was obtained by venepuncture. At the time of blood collection, patients A, O, and M had active renal disease, B and H had arthritis only, and MC had no clinical symptomatology. All patients but O had a history of elevated anti-DNA titers. Four patients, B, H, M, and MC, also had elevated F4 reactivity. A seventh patient, D, underwent splenectomy for clinical indications; therefore, splenocytes were used (6). No serum was available from this patient. Lymphocytes were obtained by Ficoll-Hypaque separation of whole blood and transformed using EBV as described previously (6, 8).

Assays for isotype and for F4 idiotype

Idiotypic reactivity was tested as follows. ELISA wells were coated with purified mouse F4 mAb at 20 µg/ml and incubated at 37°C for 1 h. After blocking (3% BSA in PBS), culture supernatants were added for 1 h at 37°C followed by peroxidase-conjugated goat anti–

Table II. Millipore Filter Assay for Binding to dsDNA

Cell line	Mean±SD	dsDNA reactivity
	cpm	
A78	45±14.4	−
B5–3	86±25.2	−
B12–2	245±59.9	+
B17–3	75±21.8	−
H17	640±41.9	++
M36	396±36.8	+
MC90	410±15.2	+
050	3047±143.6	+++
Human Ig	21±0	−

Supernatants from EBV cell lines (2 µg/ml) and human Ig (5 µg) were incubated with ^{32}P-labeled dsDNA and passed through a nitrocellulose filter. Reactivity is scored as follows: − (0–100 cpm), + (100–500 cpm), ++ (500–1,000 cpm), and +++ (> 1,000 cpm).

human IgG/IgM/IgA (Organon Teknika-Cappel, West Chester, PA). ABTS peroxidase substrate was added (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The OD was read at 405 nm on an automated ELISA reader. 3I and 8.12 reactivity were analyzed similarly, using purified 3I or 8.12 antibody adsorbed to ELISA plates.

Cells from wells containing F4+ antibody in the supernatant were cloned by limiting dilution as described previously (6), and retested for expression of the F4, 3I, and 8.12 idiotypes as described above.

Culture supernatants of F4+ clones were tested for the presence of human κ or λ and µ, γ, or α chains in a standard ELISA. Briefly, polyclonal goat anti–human IgM/IgG/IgA (heavy and light) antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) was adsorbed to ELISA plates (Falcon 3915; Becton Dickinson Labware, Lincoln Park, NJ) for 1 h at 37°C. After blocking, culture supernatants were added for 1 h at 37°C, followed by peroxidase-conjugated goat anti–human IgM/IgA/IgG κ or λ (1:1,000) (Southern Biotechnology Associates, Inc.) for 1 h at 37°C. ABTS peroxidase substrate was added, and the OD was read at 405 nm using an ELISA reader (Titertek Multiscan Plus; Ealabs, Finland).

Antigen specificity

dsDNA binding assay. Binding to dsDNA was determined by Millipore filter assay (Millipore Corp., Bedford, MA) (6). Briefly, supernatants were normalized for Ig concentration and tested for binding to plasmid dsDNA using nick-translated ^{32}P-labeled DNA made double-stranded previously by passage through a nitrocellulose filter (HAWP 45; Millipore Corp.). Supernatants were not tested for single-stranded DNA binding.

Reactivity to Smith antigen (Sm), cardiolipin, and pneumococcal polysaccharide. Direct ELISAs were performed to test for other autoantigenic specificities according to a modified protocol from Swanson et al. (9). Briefly, wells were coated with Sm (10 µg/ml) overnight at 4°C. Supernatants (5 µg/ml) were added for 45 min at 25°C. Bound samples were detected with a goat anti–human Ig (heavy and light)—alkaline phosphatase–labeled conjugate (Southern Biotechnology Associates, Inc.) and developed using 104 substrate (Sigma Chemical Co., St. Louis, MO). Binding of cardiolipin (Fluka Chemical Co., Ronkonkoma, NY) was tested by adsorbing cardiolipin, 50 µg/ml in ethanol, to Immulon II microtiter plates overnight (Dynatech Laboratories, Inc., Chantilly, VA). Samples were added for 2 h at 25°C, and bound antibody was detected as described above.
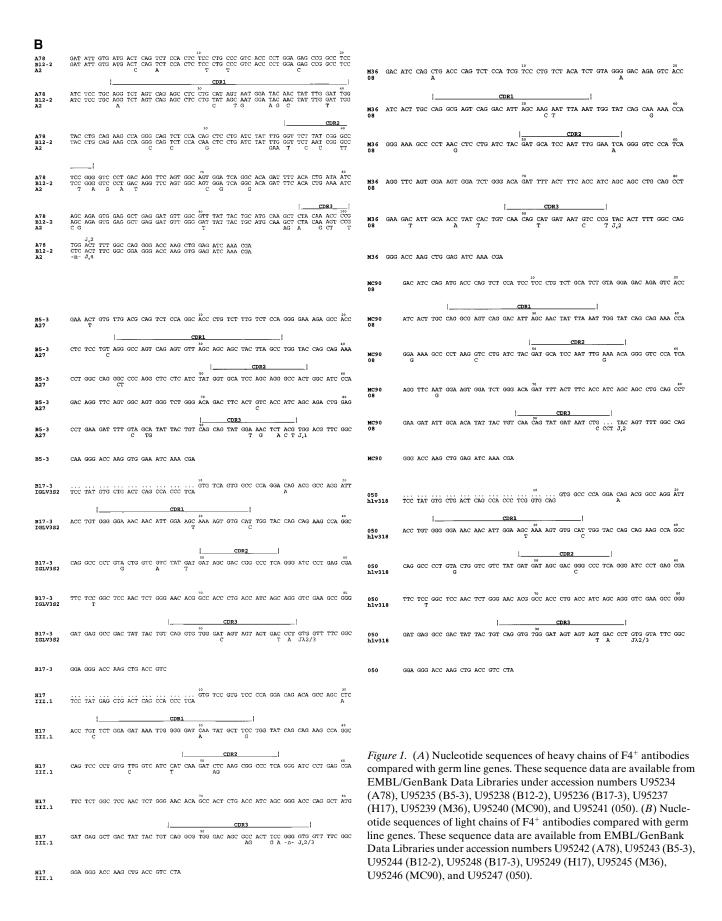
To assay binding to pneumococcal polysaccharide, 4 µg Pneumovax® 23 (Merck Sharp and Dohme, West Point, PA) was coated

A

```
A78    CAG GTG CAG CTG GTC GAG TCT GGG GGA GGC TTG GTC AAG CCT GGA GGG TCC CTG AGA CTC
DP35                            G
                                                            |___CDR1___|
A78    TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT GAC TTC TAC ATG AGT TGG ATC CGC CAG GCT
DP35                                           A               C       G
                                                                                |         CDR2
A78    CCG GGG AAG GGG CTG GAG TGG GTT TCA TAC ATT AGT AAT TGT AGT CGT TAC ACA AAC TAC
DP35                                                 G A G  A  AC  T  T
       _____|
A78    GCA GAC TCT GTG AAG GGC CGC TTC ACC ATC TCC AGA GAC AAC GCC AAC AAC TCA CTG TTT
DP35                        A                   G              G              A
A78    CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC GCG GCC GTG TAT GAC TGT GCG AGA GAG GGG
DP35                                       A       T                          D
A78    GAT ATT GTA GTA GTA CCA ACT GAG GGT GAC GTT GCC TCG AGG CGG ACT GGC TGG TTC GAC
DP35                                                   J_H5
A78    CCC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA
```

```
B5-3   CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AAG TCC CTG AGA CTC
DP49                                                                     G
                                                            |___CDR1___|
B5-3   TCC TGT GCA GCC TCT GGA TTC ACC CTC AGT TCC TAT GGC ATG CAC TGG GTC CGC CAG GCT
DP49                                   T    AG
                                                                           |        CDR2
B5-3   CCA GGC AAG GGG CTG GAG TGG GTG TCA TTT ATA CGG TAT GAT GGA ACC CAC AAA TAC TAT
DP49                                       G   G    TCA           GT A T
       _____|
B5-3   GCA GAC TCC GTG AAG GGC CAA TTC AGC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
DP49                            G       C
B5-3   CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT ATG TAT TTC TGT GCG AAA GAT CAT
DP49                                          G       A           D
B5-3   CCA GCT GGT ACT GGG AGC TTT GAC TCC TGG GGC CAG GGA ACC CTG GTC
                                J_H4
```

```
B12-2  GAG GTG CTT CTG GTG GAG TCT GGG GGA GGC CTG GTC AAG CCT GGG GGG TCC CTG AGA CTC
DP51           AG                            T    A C
                                                            |___CDR1___|
B12-2  TCC TGT GCC GCC TCT GGA TTC ATC TTA AGT AGT TAT AGC ATG AAC TGG GTC CGC CAG GCT
DP51           A                        C    C     C
                                                                           |       CDR2
B12-2  CCA GGG AAG GGG CCG GAG TGG GTC TCA TCT ATT AGT TAT AGT AGT ACT TAC ATT TCG TAC
DP51                        T           T    AC        AG          G AC   A AC
       _____|
B12-2  GCA GAC TCA GTG AAG GGC CGA TTC ACC GTT TCC AGA GAC CAC GCC AAG AAC TCA CTG TAT
DP51                   T                   A C              A T
B12-2  CTG CAA ATG GAC AGC CTG AGA GCC GAG GAC ACG GCT GTC TAT TAC TGT GCG AGA TCC CCA
DP51                   A               A               G                  D
B12-2  CTT TGC ACT TAC GAC TGC CAC TAC TAT CGT ATG GAC GTC TGG GGC CAA GGG ACC ACG GTC
                                J_H6
B12-2  ACC GTC TCC TCA
```

```
B17-3  GAG GTG CAG CTG TTA GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG GGG TCC CTG AGA CTC
DP47                            G
                                                            |___CDR1___|
B17-3  TCC TGT GTA GCC TCT GGA TTC TCC TTT AGC CGC TCT GCC ATG GCC TGG GTC CGC CAG GCT
DP47           C                        A           A   A       AG
                                                                          |        CDR2
B17-3  CCA GGG AAG GGG CTG GAG TGG GTC TCA GCT ATT AGT GGT ATT GAT GAT GTC ACA TAC TAC
DP47                                               G   G   G AG
       _____|
B17-3  GCA GGC TCC GTG AAG GGC CGG TTC ACC ATC TCC AGA GAC AAT TCC AAG AGC ACG TTG TAT
DP47           A                                                       A       C
B17-3  CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCC CTA TAT TAC TGT GCG AGG GAT TCT
DP47                                       G                           AA D
B17-3  TTG TGG GAA TAT AGT TAT GGT CCC AAG AAC TTT GAC CTC TGG GGC CAG GGA ACG CTG GTC
                                    J_H4
B17-3  ACC
```

```
H17    GAG GTT CAA CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG GGG TCC CTG AGA CTC
DP47         G  G
                                                            |___CDR1___|
H17    TCC TGT GCA GCC TCT GGA TTC ACC TTT AGC AGC TAT GCC ATG ACC TGG GTC CGC CAG GCT
DP47                                                       G
                                                                           |        CDR2
H17    CCA GGG AAG GGG CTG GAG TGG GTC CCA AGA ATT AGT GGT CGT GGT GGT AGC ACA TAC TAC
DP47                                   T   GCT                 A
       _____|
H17    GCA GAC TCC GTG AAG GGC CGG TTC ACC ATC TCC AAA GAC AAT TCC AAG AAC ACG CTG TAT
DP47                                               G
H17    CTG CAA ATG AAC AGC CTG AGA GCC GAC GAC GCC GTA TAT TAC TGT GCG CGG TCG AGT
DP47                                   G   A G                        AAA D
H17    AGT GCC TAT TAC GGT ATT ATG ACT CAT TAT TAT AGC TTT GAC TAC TGG GGC CAG GGA ACC
DP47                                    J_H4
H17    CTG GTC ACC GTC TCA
```

```
M36    CAA GTC CAA CTG GTG CAG TCT GGG GCT GAA GTG AAG AAG CCT GGG TCC TCG GTA AAG GTC
hv1263     G  G                                       G                          G
                                                            |___CDR1___|
M36    TCC TGC AAG GCT TCC GGA GGC ACC TTC AAC AAC TAT GCT GTC AAC TGG ATG CGG CAG GCC
hv1263                         T       G  G        A  G        G  A
                                                                           |      CDR2
M36    CCT GGA CAA GGG CTT GAG TGG ATG GCA AGG ATC ATC CCT CAC CTT GAT GTA AAA AAT ACC
hv1263                                   G             AT        G A GC    C TA
       _____|
M36    GCA CAG AGG TTC GAG GAC AGA GTC ACG ATT ACC GCG GAC ACA TCG ACG AGC ACA GTC TTC
hv1263             A   C   G               A           A   C               C A
M36    ATG GAA CTG AGC AGC CTG AGA TCT GAA GAC ACG GCC GTG TAT TAC TGT GCG AGA CGA AAG
hv1263             G                   G                                       D
M36    AAG GAG TGG CTG ATA CCC GGG GCT TTT GAT CTC TGG GGC CAA GGG ACA ATG GTC ACC GTC
hv1263                             J_H3
M36    TCT TCA
```

```
MC90   GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGA GGG TCC CTG AGA CTC
DP58
                                                            |___CDR1___|
MC90   TCC TGT GGA GCC TCT GGA TTC ACC TTT AGT AGT TAT GAA ATG AAC TGG GTC CGC CAG GCT
DP58           C                        C           C
                                                                           |       CDR2
MC90   CCA GGG AAG GGG CTG GAG TGG CTT TCA TAC ATT AGT CGT AGT GGT GAT ACC ATA TAC TAT
DP58                           G            A           AG                       C
       _____|
MC90   GCA GAC TCT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC TCA CTG TTT
DP58                                                                           A
MC90   CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG GCT GTT TAT TAC TGT GCG AGA GGC CCC
DP58                                   T               G                      D
MC90   CGC TAC GAA GGG CCG TAC TAC TAC TTT GAC TAC TGG GGC CAG GGA ACG CTG GTC ACC GTC
DP58                             J_H4
MC90   TCC TCA
```

```
O50    CAG GTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAG ACC CTG TCC CTC
V71-4
                                                            |___CDR1___|
O50    ACC TGC ACT GTC TCT AGT GGC TCC ATC AGT AGT CAC TAC TGG AAC TGG ATC CGG CAG ACC
V71-4                             G               G        T    G                  C
                                                                           |     CDR2
O50    CCA GGG AAG GGG CTG GAA TGG ATT GGG AAT ATC TAT TAC AGT GGG AGC ACC AAC TAC AAC
V71-4                             A   G       T
       _____|
O50    CCC TCC CTC AAG AGT CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG AAG CAG TTC TCC CTG
V71-4                                                                   C
O50    AAG GTG AGC TCT GTG ACC TCT GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA GAG GCA GCA
V71-4      C                       G                                       D
O50    GCT GGT TAT GAT GCT TTT GAT ATC TGG GGC CAA GGG ACA ATG GTC ACC GTC TCT TCA
                        J_H3
```

on ELISA immunoadsorbant assay/RIA high-binding plates (Costar Corp., Cambridge, MA) overnight at 4°C. After washing with PBS-Tween (0.05%) and blocking (3% BSA in PBS), supernatants were added for 1 h at 37°C, and the assay proceeded as described above.

Total RNA preparation

Total RNA was prepared from cell lines using guanidinium thiocyanate (6) or the Snap-O-Sol nucleic acid preparation kit (Biotecx Laboratories, Houston, TX). Northern blot analysis (6) was used to verify

B

```
                                                                              10                                        20
A78    GAT ATT GTG ATG ACT CAG TCT CCA CTC TCC CTG CCC GTC ACC CCT GGA GAG CCG GCC TCC
B12-2  GAT ATT GTG ATG ACT CAG TCT CCA CTC TCC CTG CCC GTC ACC CCT GGA GAG CCG GCC TCC
A2                             C     A                                      C
                         |_____CDR1_____|
                                                            30                                  40
A78    ATC TCC TGC AGG TCT AGT CAG AGC CTC CTG CAT AGT AAT GGA TAC AAC TAT TTG GAT TGG
B12-2  ATC TCC TGC AGG TCT AGT CAG AGC CTC CTG CAT AGT AAT GGA TAC AAC TAT TTG GAT TGG
A2                A                              C   T G        A G C           T
                                                                   |_____CDR2_____
                                         50                                  60
A78    TAC CTG CAG AAG CCA GGG CAG TCT CCA CAG CTC CTG ATC TAT TTG GGT TCT TAT CGG GCC
B12-2  TAC CTG CAG AAG CCA GGG CAG TCT CCA CAG CTC CTG ATC TAT TTG GGT TCT TAT CGG GCC
A2                            C   C        G                   GAA T   C   C     TT
       _____|
                                             70                                  80
A78    TCC GGG GTC CCT GAC AGG TTC AGT GGC AGT GGA TCA GGC ACA GAT TTT ACA CTG ATA ATC
B12-2  TCC GGG GTC CCT GAC AGG TTC AGT GGC AGT GGA TCA GGC ACA GAT TTC ACA CTG AAA ATC
A2        T   A   G A   T                          C   G        G
                                                              |_____CDR3_____|
                                         90                        100
A78    AGC AGA GTG GAG GCT GAG GAT GTT GGC GTT TAT TAC TGC ATG CAA GCT CTA CAA ACC CCG
B12-2  AGC AGA GTG GAG GCT GAG GAT GTT GGG GAT TAT TAC TGC ATG CAA GCT CTA CAA AGT CCG
A2     C G                              T                        AG A     G CT     T
              J.2
A78    TGG ACT TTT GGC CAG GGG ACC AAG CTG GAG ATC AAA CGA
B12-2  CTC ACT TTC GGC GGA GGG ACC AAG GTG GAG ATC AAA CGA
A2     -n- J.4
```

```
                                                            10                                  20
B5-3   GAA ACT GTG TTG ACG CAG TCT CCA GGC ACC CTG TCT TTG TCT CCA GGG GAA AGA GCC ACC
A27        T
                         |_____CDR1_____|
                                            30                                  40
B5-3   CTC TCC TGT AGG GCC AGT CAG AGT GTT AGC AGC AGC TAC TTA GCC TGG TAC CAG CAG AAA
A27                 C
                                 |_____CDR2_____|
                            50                                  60
B5-3   CCT GGC CAG GGC CCC AGG CTC CTC ATC TAT GGT GCA TCC AGC AGG GCC ACT GGC ATC CCA
A27               CT
                                            70                                  80
B5-3   GAC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT GTC ACC ATC AGC AGA CTG GAG
A27                                                    C
                         |_____CDR3_____|
                                            90
B5-3   CCT GAA GAT TTT GTA GCA TAT TAC TGT CAG CAG TAT GGA AAC TCT ACG TGG ACG TTC GGC
A27               C   TG                    T   G         A C T J.1

B5-3   CAA GGG ACC AAG GTG GAA ATC AAA CGA
```

```
                                                                         10                        20
B17-3    ... ... ... ... ... ... ... ... ... GTG TCA GTG GCC CCA GGA CAG ACG GCC AGG ATT
IGLV3S2  TCC TAT GTG CTG ACT CAG CCA CCC TCA                                      A
                         |_____CDR1_____|
                                                   30
B17-3    ACC TGT GGG GGA AAC AAC ATT GGA AGC AAA AGT GTG CAT TGG TAC CAG CAG AAG CCA GGC
IGLV3S2                             T               C
                                 |_____CDR2_____|
                            50                                  60
B17-3    CAG GCC CCT GTA CTG GTC GTC TAT GAT AGC GAC CGG CCC TCA GGG ATC CCT GAG CGA
IGLV3S2              G       A       T
                                             70                                  80
B17-3    TTC TCC GGC TCC AAC TCT GGG AAC ACG GCC ACC CTG ACC ATC AGC AGG GTC GAA GCC GGG
IGLV3S2      T
                         |_____CDR3_____|
                                             90
B17-3    GAT GAG GCC GAC TAT TAC TGT CAG GTG TGG GAT AGT AGT AGT GAC CCT GTG GTT TTC GGC
IGLV3S2                                  C              T   A  Jλ2/3

B17-3    GGA GGG ACC AAG CTG ACC GTC
```

```
                                                                         10                        20
H17     ... ... ... ... ... ... ... ... ... GTG TCC GTG TCC CCA GGA CAG ACA GCC AGC CTC
III.1   TCC TAT GAG CTG ACT CAG CCA CCC TCA                                      A
                        |_____CDR1_____|
                                                  30
H17     ACC TGT TCT GGA GAT AAA TTG GGG GAT CAA TAT GCT TCC TGG TAT CAG CAG AAG CCA GGC
III.1          C                        A           G
                                 |_____CDR2_____|
                            50
H17     CAG TCC CCT GTG TTG GTC ATC CAT CAA GAT CTC AAG CGG CCC TCA GGG ATC CCT GAG CGA
III.1                          C       T       AG
                                             70                                  80
H17     TTC TCT GGC TCC AAC TCT GGG AAC ACA GCC ACT CTG ACC ATC AGC GGG ACC CAG GCT ATG
III.1
                        |_____CDR3_____|
                                             90
H17     GAT GAG GCT GAC TAT TAC TGT CAG GCG TGG GAC AGC GCC ACT TCC GGG GTG GTT TTC GGC
III.1                                  AG       G A -n- J.2/3

H17     GGA GGG ACC AAG CTG ACC GTC CTA
```

```
                                                                     10                                        20
M36    GAC ATC CAG CTG ACC CAG TCT CCA TCG TCC CTG TCT ACA TCT GTA GGG GAC AGA GTC ACC
08                 A                                                          A
                         |_____CDR1_____|
                                                   30
M36    ATC ACT TGC CAG GCG AGT CAG GAC ATT AGC AAG AAT TTA AAT TGG TAT CAG CAA AAA CCA
08                                          C T                              G
                                 |_____CDR2_____|
                            50                                  60
M36    GGG AAA GCC CCT AAC CTC CTG ATC TAC GAT GCA TCC AAT TTG GAA TCA GGG GTC CCA TCA
08                     G                                           A
                                             70                                  80
M36    AGG TTC AGT GGA AGT GGA TCT GGG ACA GAT TTT ACT TTC ACC ATC AGC AGC CTG CAG CCT
08
                                 |_____CDR3_____|
                                             90
M36    GAA GAC ATT GCA ACC TAT CAC TGT CAA CAG CAT GAT AAT GTC CCG TAC ACT TTT GGC CAG
08         T          A   T              T                  C   T J.2

M36    GGG ACC AAG CTG GAG ATC AAA CGA
08
```

```
                                                                     10                                        20
MC90   GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA GAC AGA GTC ACC
08
                         |_____CDR1_____|
                                                   30                                  40
MC90   ATC ACT TGC CAG GCG AGT CAG GAC ATT AGC AAC TAT TTA AAT TGG TAT CAG CAG AAA CCA
08
                                 |_____CDR2_____|
                            50                                  60
MC90   GGA AAA GCC CCT AAG GTC CTG ATC TAC GAT GCA TCC AAT TTG AAA ACA GGG GTC CCA TCA
08         G                  C                                   G
                                             70                                  80
MC90   AGG TTC AAT GGA AGT GGA TCT GGG ACA GAT TTT ACT TTC ACC ATC AGC AGC CTG CAG CCT
08         G
                                 |_____CDR3_____|
                                             90
MC90   GAA GAT ATT GCA ACA TAT TAC TGT CAA CAG TAT GAT AAT CTG ... TAC AGT TTT GGC CAG
08                                                          C CCT J.2

MC90   GGG ACC AAG CTG GAG ATC AAA CGA
```

```
                                                                         10                        20
050    ... ... ... ... ... ... ... ... ... ... GTG GCC CCA GGA CAG ACG GCC AGG ATT
hlv318 TCC TAT GTG CTG ACT CAG CCA CCC TCG GTG CAG                         A
                         |_____CDR1_____|
                                                   30                                  40
050    ACC TGT GGG GGA AAC AAC ATT GGA AGC AAA AGT GTG CAT TGG TAC CAG CAG AAG CCA GGC
hlv318                             T               C
                                 |_____CDR2_____|
                            50                                  60
050    CAG GCC CCT GTA CTG GTC GTC TAT GAT GAT AGC GAC GGG CCC TCA GGG ATC CCT GAG CGA
hlv318              G                                 C
                                             70                                  80
050    TTC TCC GGC TCC AAC TCT GGG AAC ACG GCC ACC CTG ACC ATC AGC AGG GTC GAA GCC GGG
hlv318     T
                                 |_____CDR3_____|
                                             90
050    GAT GAG GCC GAC TAT TAC TGT CAG GTG TGG GAT AGT AGT GAC CCT GTG GTA TTC GGC
hlv318                                              T   A  Jλ2/3

050    GGA GGG ACC AAG CTG ACC GTC CTA
```

Figure 1. (*A*) Nucleotide sequences of heavy chains of F4+ antibodies compared with germ line genes. These sequence data are available from EMBL/GenBank Data Libraries under accession numbers U95234 (A78), U95235 (B5-3), U95238 (B12-2), U95236 (B17-3), U95237 (H17), U95239 (M36), U95240 (MC90), and U95241 (050). (*B*) Nucleotide sequences of light chains of F4+ antibodies compared with germ line genes. These sequence data are available from EMBL/GenBank Data Libraries under accession numbers U95242 (A78), U95243 (B5-3), U95244 (B12-2), U95248 (B17-3), U95249 (H17), U95245 (M36), U95246 (MC90), and U95247 (050).

heavy chain isotype and variable region gene family using Cμ (5′-GAGGGGGAAAAGGGTTGGGGC-3′) and Cγ (5′-GCCAGG-GGGAAGACCGATGG-3′) oligonucleotides and genomic DNA probes representing heavy (V_H) and light (V_L) chain variable gene families (6).

Sequence of heavy and light chain V region genes

5 μg total RNA was reverse-transcribed using a Cγ oligomer and Superscript® II RNase H reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Heavy chain variable region sequences were amplified with Vent polymerase (New England Biolabs Inc., Beverly, MA) by PCR using the appropriate 3′ constant region oligomer in conjunction with 5′ leader oligomers for V_H1 (5′-ATGGACTGGACCTG-GCAGGAGTC-3′), V_H3 (5′-ATGGAGTTTGGGCTGAGCTGG-3′), or V_H4 (5′-ATGAAACACCTGTGGTTCTTC-3′). PCR DNA products were recovered by the Spin Bind PCR purification system (FMC BioProducts, Rockland, ME). Light chains were amplified similarly using a universal Cλ (5′-AGTGTGGCCTTGTTGGCTTG-3′) or Jλ 2/3 (5′-TAGGACGGTCAGCTTGGTCCCTCCGCCGAAAAC-CAC-3′) or a Cκ (5′-GTTCCAGATTTCAACTGCTC-3′) oligomer and either 5′ leader oligomers for $V_κI$ (5′-CATGAGGGTCC-CCGCTCAG-3′), $V_κII$ (5′-ATGAGGCTCCCTGCTCAGCTC-3′), or $V_κIII$ (5′-ATGGAAACCCCAGCGCAGCT-3′), or a 5′ framework region (FR) 1 Vλ3 (5′-TCTGTGGAGCTCCAGCCGCCCT-CAGTG-3′) oligomer.

PCR products were cloned into the TA cloning vector pCR® II (Invitrogen Corp., San Diego, CA), while Cλ products were cloned into pBluescript II or pCR script vectors (Stratagene Inc., La Jolla, CA). All were sequenced using Sequenase version 2 (USB Biologicals, Cleveland, OH). The University of Wisconsin Genetics Computer Group was used to analyze amino acid sequences as well as to compute isoelectric points. The predicted replacement (R) to silent (S) mutation ratios were determined by a computer-generated program (rsanal) developed by Joseph Mindell, Daniel Lustgarten, and Elahna Paul (Albert Einstein College of Medicine). This program analyzes the likelihood that point mutation of each nucleotide in the V region will lead to an R or S substitution. Therefore, each nucleotide is hypothetically changed to each of the other three nucleotides, and the cumulative R/S ratio is determined.

Statistics

RGYW (R = A/G, Y = T/C, W = A/T) or trinucleotide hot spots were identified using a computer-generated program. The targeting of silent mutations to hot spots was determined using 2 × 2 contingency tables (Statview computer program). *P* values are given for Pearson's χ^2 analysis.

Results

Generation and characterization of F4+ lines. Sera from six lupus patients were titered for dsDNA reactivity. High-titered dsDNA reactivity (greater than the mean of control sera +2SD) was observed for patients A, B, H, M, and MC (data not shown), but not for patient O. Similarly, elevated levels of F4 were observed in sera from all patients except patients O and H (data not shown). Peripheral blood lymphocytes from these patients were transformed with EBV and cloned at 1 wk, before screening for idiotype expression. In addition, splenocytes from patient D were transformed by EBV. No simultaneous serum was available for idiotypic analysis. Approximately 100 clones from each patient were screened. 21 independent idiotype-positive clones were identified, and 8 of these were maintained successfully in culture. The characteristics of these eight clones as well as two F4+ antibodies (I-2a and H2F) described previously are shown in Table I. All antibodies express an IgG$_1$ heavy chain (data not shown). This is

consistent with previous studies of myeloma proteins and serum Ig showing that F4+ Igs are almost exclusively IgG (3). Seven lines express a κ light chain, all of which are 3I+, confirming the preferential association of F4+ heavy chains with 3I+ light chains (3). Three lines, each derived from a different patient, express a λ light chain, although none is 8.12+. This observation is also consistent with studies of myeloma proteins showing no association between F4 and 8.12 specificities.

F4+ antibodies were tested for binding to dsDNA and a panel of SLE autoantigens with which anti-DNA antibodies may cross-react. Five of the eight novel F4+ antibodies as well as the two antibodies reported previously (6) bound dsDNA, confirming the strong association of F4 reactivity with native DNA (Table II). None bound Sm or cardiolipin (data not shown). The antibodies were also tested for binding to pneumococcal polysaccharide, since we had shown previously that antibodies expressing anti-DNA–associated idiotypes may bind pneumococcal antigen. None was reactive to pneumococcal polysaccharide by ELISA (data not shown).

V gene usage in F4+ antibodies. Of the 10 F4+ antibodies, 8 have heavy chains encoded by members of the V_H3 gene family, with 3 of these deriving from the DP47 germ line gene. One antibody is encoded by a member of the V_H1 gene family, and another is encoded by a gene from the V_H4 family (Table III) (10–13). All V_H genes have been reported to be expressed in non–DNA-binding antibodies from nonautoimmune individuals. Nucleotide sequences of the eight novel F4+ variable regions are displayed in Fig. 1 *A*.

7 of the 10 light chains are encoded by V κ genes from the $V_κI$, $V_κII$, $V_κIII$, and $V_κIV$ families. The three antibodies expressing λ light chains all use Vλ3 genes. Nucleotide sequences of novel F4+ light chains are shown in Fig. 1 *B*. A FASTA computer search was used to identify the germ line genes most homologous to the genes encoding the expressed F4+ lupus antibodies. Nucleic and amino acid homologies to putative germ line genes are presented in Table III.

Protein sequences of F4+ antibodies shown in Figs. 2 and 3 were compared to the protein sequences encoded by the putative germ line genes. Arginines in complementarity-determining region (CDR) 3 of the heavy chain have been suggested to be an important structural motif of murine DNA-binding antibodies (14). In addition, asparagine, lysine, and glutamine have been implicated in DNA binding (15). While there was a notable absence of arginines in CDR3 of F4+ heavy chains, arginine, asparagine, and charged residues were present frequently in CDR1 and CDR2, often representing an amino acid substitution from the germ line encoded sequence.

Since nephritogenic antibodies are often cationic (16–18), it was of interest to determine the isoelectric points of the heavy and light chain variable region sequences. Most F4 reactive heavy chains are cationic; only two are not, and these two derive from non–DNA-binding antibodies (Table IV). In general, the germ line encoded sequence is strongly cationic, and mutation is not required to generate the charge of the heavy or light chain variable region.

Mutational analysis. Extensive somatic mutation is apparent in many of the heavy chain V region sequences (Fig. 1 *A*), consistent with, although not proving, the hypothesis that mutation is necessary for expression of the F4 idiotype. All light chain V region genes are also mutated somatically (Fig. 1 *B*). Since the heavy chains express the SLE-specific F4 idiotype, these were analyzed extensively to see if they might provide
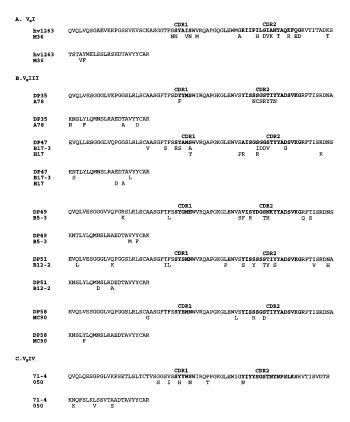
A. V_H I

```
                                       CDR1                    CDR2
   hv1263   QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILGTIANYAQKFQGRVTITADKS
   M36                        NN  VN M           A    H DVK T R ED      T

   hv1263   TSTAYMELSSLRSEDTAVYYCAR
   M36         VF
```

B. V_H III

```
                                        CDR1                    CDR2
   DP35    QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYMSWIRQAPGKGLEWVSYISSSGSTIYYADSVKGRFTISRDNA
   A78                                  F                    NCSRYTN

   DP35    KNSLYLQMNSLRAEDTAVYYCAR
   A78     N  F        A D

                                        CDR1                    CDR2
   DP47    EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTIYYADSVKGRFTISRDNS
   B17-3              V   S RS A                             IDDV   G
   H17                          T                       PR  R                      K

   DP47    KNTLYLQMNSLRAEDTAVYYCAR
   B17-3   S          L
   H17              D A

                                        CDR1                    CDR2
   DP49    QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNS
   B5-3               K           L                        SF R  TH       Q S

   DP49    KNTLYLQMNSLRAEDTAVYYCAK
   B5-3                M F

                                        CDR1                    CDR2
   DP51    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMNWVRQAPGKGLEWVSYISSSSSTIYYADSVKGRFTISRDNA
   B12-2   L     K           IL                       P   S Y TY S        V  H

   DP51    KNSLYLQMNSLRDEDTAVYYCAR
   B12-2        D  A

                                        CDR1                    CDR2
   DP58    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYEMNWVRQAPGKGLEWVSYISSSGSTIYYADSVKGRFTISRDNA
   MC90                 G                              L  R D

   DP58    KNSLYLQMNSLRAEDTAVYYCAR
   MC90       F
```

C. V_H IV

```
                                        CDR1                    CDR2
   71-4    QVQLQESGPGLVKPSETLSLTCTVSGGSVSSYYWSWIRQPPGKGLEWIGYIYYSGSTNYNPSLKSRVTISVDTS
   050                           S  I H N  T             N

   71-4    KNQFSLKLSSVTAADTAVYYCAR
   050     K   V   S
```

Figure 2. The deduced protein sequences of heavy chains of F4[+] antibodies compared with translated germ line genes.

evidence of lupus-specific mutational processes. The R/S mutation ratios were computed over the FRs and CDRs for each variable region (Table V). Clustering of R mutations in CDRs and a higher than random R/S ratio in CDRs are both considered evidence of antigen selection. R mutations were clustered in CDRs of F4[+] antibodies; 70% of all R mutations were in CDRs. While the R/S ratios in CDR1 were lower than the predicted random ratios, F4[+] heavy chains displayed a trend for higher than random R/S ratios in CDR2.

We sought to compare the frequency of mutation in F4[+] heavy chain V_H genes to that in protective antibodies encoded by the same V_H genes derived from nonautoimmune individuals (Table VI) (19–29). No significant difference between the two groups was found; F4[+] V_H genes contained 194 mutations in 10 sequences, while 437 mutations were present in 24 sequences from nonautoimmune individuals (data not shown).

Several investigators have described mutational hot spots, which appear to be targets of hypermutation (30–32). Rogozin and Kolchanov (31) have identified sequences RGYW as potential hot spots. Recently, Smith et al. analyzed the untranslated flanking regions of Ig V genes and concluded that four trinucleotide sequences, AGC, TAC, GCT, and GTA, appear to be dominant mutational hot spots (32). We were interested in determining whether these hot spots were targeted for mutation in SLE B cells. Therefore, we examined the frequency of mutation in hot spots (mutated/nonmutated) compared with the frequency of mutation in the rest of the gene in both SLE and non-SLE antibodies. To insure that the analysis was not confounded by altered selection in SLE, we analyzed only

S mutations, which could not be subject to pressures of selection. The data show that there is a significant targeting of mutation to RGYW hot spots in antibodies derived from non-SLE individuals. 34 of 1,164 hot spots were mutated; in contrast, only 64 of 5,979 non–hot spots were mutated ($P < 0.0001$, Table VII). This is in striking contrast to SLE antibodies, in which there was no significant targeting of mutation to RGYW motifs. 13 of 485 hot spots displayed S mutations, while 42 of 2,507 non–hot spots displayed S mutations ($P > 0.1$, Table VII). When trinucleotide hot spots were examined, there again was a significant clustering of S mutations in hot spot motifs in antibodies from nonautoimmune individuals ($P < 0.0001$, Table VII). In SLE antibodies, targeting to trinucleotide hot spots was also evident but was less than that observed for non-SLE antibodies ($P < 0.01$, Table VII).

Discussion

The molecular genetic analysis of EBV-transformed F4^{+} cells confirms the results of previous studies on F4[+] myeloma proteins and SLE sera (3). All of the F4[+] antibodies reported here are IgG$_1$, confirming the observation that F4 reactivity is highly restricted to IgG. Second, there is a strong correlation between F4[+] expression, cationicity, and DNA binding. In fact, our results show that all but two F4[+] heavy chains in this study are cationic, and of the eight cationic F4[+] heavy chains, seven bind dsDNA. Sequence analysis revealed that the germ
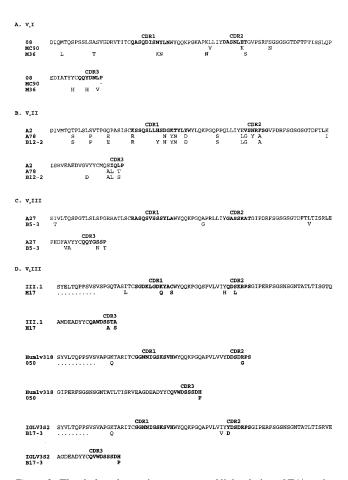
A. V_L I

```
                                          CDR1                  CDR2
   08      DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQP
   MC90                                                       V        K     N
   M36        L       T                   KN           N         S

                                          CDR3
   08      EDIATYYCQQYDNLP
   MC90                  -
   M36          H   H V
```

B. V_L II

```
                                            CDR1                    CDR2
   A2      DIVMTQTPLSLSVTPGQPASISCKSSQSLLHSDGKTYLYWYLQKPGQPPQLLIYEVSNRFSGVPDRFSGSGSGTDFTLK
   A78               S    P    E    R        N YN  D        S      LG Y A                    I
   B12-2             S    P    E    R        YN YN D        S      LG   A

                                         CDR3
   A2      ISRVEAEDVGVYYCMQSIQLP
   A78                      AL T
   B12-2           D        AL S
```

C. V_L III

```
                                            CDR1                    CDR2
   A27     EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLE
   B5-3    T                                              G                              V

                                         CDR3
   A27     PEDFAVYYCQQYGSSP
   B5-3    VA        N T
```

D. V_L III

```
                                            CDR1                    CDR2
   III.1   SYELTQPPSVSVSPGQTASITCSGDKLGDKYACWYQQKPGQSPVLVIYQDSKRPSGIPERFSGSNSGNTATLTISGTQ
   H17     ...........          L    Q S              H L

                                         CDR3
   III.1   AMDEADYYCQAWDSSTA
   H17               A S

                                               CDR1                CDR2
   Humlv318  SYVLTQPPSVSVAPGKTARITCGGNNIGSKSVHWYQQKPGQAPVLVVYDDSDRPS
   050       ...........          Q                          G

                                            CDR3
   Humlv318  GIPERFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSSDH
   050                                       P

                                               CDR1                CDR2
   IGLV3S2  SYVLTQPPSVSVAPGKTARITCGGNNIGSKSVHWYQQKPGQAPVLVIYYDSDRPSGIPERFSGSNSGNTATLTISRVE
   B17-3    .........            Q                          V D

                                            CDR3
   IGLV3S2  AGDEADYYCQVWDSSSDH
   B17-3              P
```

Figure 3. The deduced protein sequences of light chains of F4[+] antibodies compared with translated germ line genes.

Cell line	Germ line V_H	Heavy chain VDJ	Germ line V_L	Light chain VJ
A78	9.32	6.48	7.17	5.81
B5–3	9.87	9.31	6.45	8.66
B12–2	8.07	7.79	7.17	5.04
B17–3	8.65	6.52	5.63	4.41
H17	8.65	9.28	4.36	4.51
M36	10.25	9.34	3.94	5.65
MC90	8.07	10.29	3.94	6.44
050	9.30	8.08	4.98	4.41
I–2a	9.92	8.65	8.07	8.65
H2F	8.65	8.41	7.04	11.15

line genes used to encode F4$^+$ antibodies encode cationic sequences, and that somatic mutation is not critical for their charge. Similarly, Harada et al. have found that the very cationic light chains found in anti-DNA antibodies are encoded by a V_κ gene which in its germ line configuration encodes a highly cationic sequence (17). Furthermore, an isolated heavy chain encoded by a V_H4 variable region gene has been shown to bind dsDNA, and this heavy chain also derives from a germ line gene that is cationic (33). Antibody 050 has seven of nine CDR2 residues in common with this isolated V_H4 heavy chain.

Since F4 is a lupus-associated idiotype (3) (60% of lupus patients have elevated titers of F4$^+$ Igs in their sera), it was possible that it would be encoded by a single V_H gene. However, sequence analysis showed that several V_H gene families can encode F4$^+$ heavy chains. The fact that most of the antibodies reported here are encoded by V_H3 genes probably does not reflect an idiotypic bias for V_H3, but rather the predominant use of V_H3 genes in human peripheral blood B cells (34). Furthermore, the V_H genes used to encode F4$^+$ antibodies are expressed in the repertoire of nonautoimmune individuals, suggesting that the F4 idiotype is generated by convergent patterns of somatic mutation and not by usage of a single V_H gene or by an infrequently used subset of V_H genes.

No cell line expresses a J_H1 or J_H2 gene segment. While it is tempting to speculate that the absence of J_H1 and J_H2 suggests

that receptor editing of heavy chain genes occurs frequently in individuals with SLE, studies of 34 antibodies to foreign antigens derived from nonautoimmune individuals also show that only one anti-HIV antibody (29) and one antibody-specific *Haemophilus influenzae* type B (22) are encoded by a J_H1 or J_H2 segment. Previous analysis of 97 autoantibodies (35) showed that only 3 antibodies are encoded by J_H1 or J_H2 gene segments. The absence of these gene segments appears to be a general characteristic of the human heavy chain repertoire, perhaps due to recombination signal sequences that do not favor rearrangement. Further studies of unexpressed VDJ sequences are needed to address this question.

While F4$^+$ heavy chains can associate with λ light chains, most F4-reactive antibodies possess κ light chains, and all of these are 3I$^+$. This confirms a previous analysis of myeloma proteins that demonstrated a significant association of F4 and 3I reactivity. We looked for indirect evidence of receptor editing by examining whether any V_κ sequences were encoded by upstream V_κ segments (J_κ distal) and downstream J_κ segments. Three F4$^+$ antibodies (A78, M36, and MC90) use upstream V_κ genes; however, these do not express downstream J_κ segments. Only the B12-2 non–DNA-binding antibody expresses a J_κ–distal V_κ with a downstream J_κ segment ($J_\kappa4$). This result concurs with those of others suggesting that there is little evidence for receptor editing of light chains in these autoreactive B cells (36). Perhaps it is a lack of editing that helps predispose some individuals to the production of autoreactive B cells. In support of this argument are data showing the failure of SLE B cells to edit the A30–$J_\kappa2$–C_κ rearrangement, which is rarely expressed in nonautoimmune individuals but has been shown to exhibit nephritogenic potential in SLE individuals (37).

Table V. R/S Ratios in F4$^+$ V_H Regions

	Actual R/S ratio					Predicted random rates*				
	FR1	CDR1	FR2	CDR2	FR3	FR1	CDR1	FR2	CDR2	FR3
A78	0:1	1:1	1:0	7:0	4:2	2.4	12.7	2.9	3.9	3.0
B5–3	2:0	0:1	1:0	4:0	4:0	2.4	6.2	2.9	3.4	3.1
B12–2	4:3	0:1	1:1	5:2	4:1	2.4	10.3	2.9	4.2	3.3
B17–3	2:1	3:0	0:0	5:0	3:1	2.5	6.2	2.9	3.1	3.1
H17	0:2	1:0	1:0	2:0	4:0	2.5	6.2	2.9	3.1	3.1
M36	1:5	3:0	2:1	8:1	3:3	2.6	4.4	3.1	3.2	3.2
MC90	1:1	1:0	1:0	2:1	1:2	2.4	16.0	2.9	3.9	3.0
O50	2:0	2:0	1:2	1:0	3:0	2.3	8.6	2.9	3.5	2.6
I–2a	2:4	2:1	1:1	6:1	3:4	2.4	6.2	2.9	3.4	3.1
H2F	2:4	2:1	1:1	6:1	3:4	2.5	6.2	2.9	3.1	3.1

*Predicted random rate was determined by a computer-generated program (rsanal) developed by Joseph Mindell, Daniel Lustgarten, and Elahna Paul (Albert Einstein College of Medicine).

Table VI. Antibodies to Foreign Antigens

Antibody	Specificity	Germ line	Reference
M20003	Cytomegalovirus	hv1263	19
L08086	Rabies	hv1263	20
L08082	Rabies	DP35	20
mAb52	Rabies	DP35	20
L08088	Rabies	DP47	20
L08083	Rabies	DP47	20
L08090	Rabies	DP47	20
L25292	HIV	DP47	21
M86597	*H. influenzae* type b polysaccharide	DP47	22
M86601	*H. influenzae* type b polysaccharide	DP47	22
L14820	*H. influenzae* type b polysaccharide	DP47	22
L14822	*H. influenzae* type b polysaccharide	DP47	22
L14821	*H. influenzae* type b polysaccharide	DP47	22
X56526	*H. influenzae* type b polysaccharide	DP47	22
U27189	*Cryptococcus*	DP47	23
L26898	Cytomegalovirus	DP51	24
L04329	*Staphylococcus* A	DP51	25
L26907	Herpes	DP51	26
L38561	HIV	DP58	27
L03824	*Staphylococcus* A	DP58	25
L03825	*Staphylococcus* A	DP58	25
L03677	Hepatitis B	71–4	28
M67503	HIV	71–4	29
L08087	Rabies	71–4	20

Table VII. Silent Mutations in Hot Spots

RGYW motif	Hot spots	Non–hot spots
Non-SLE		
Mutated	34	64
Unmutated	1132	5915
Total	1164	5979 ($P < 0.001$)
SLE		
Mutated	13	42
Unmutated	472	2465
Total	485	2507 ($P > 0.1$)
Trinucleotide motifs		
Non-SLE		
Mutated	36	62
Unmutated	1425	5622
Total	1461	5684 ($P < 0.0001$)
SLE		
Mutated	19	36
Unmutated	579	2385
Total	598	2421 ($P < 0.01$)

P values are determined by a χ^2 analysis of S mutations in hot spots over total hot spots, compared with S mutations in non–hot spots over total non–hot spots. This represents the targeting of mutations to hot spot motifs.

Sequence analysis confirms the hypothesis that F4[+] antibodies are mutated somatically. In the heavy chain variable regions, mutations in CDR2 are more prevalent than mutations in CDR1. This is consistent with other studies showing an increased frequency of somatic mutation in CDR2 of V_H3 and V_H4 genes encoding autoantibodies (18, 38). Radic and Weigert have suggested that CDR2 of the heavy chain plays an important role in DNA binding, and a large number of murine anti-DNA antibodies also display extensive mutation of CDR2 in the heavy chain V region (39).

Because F4[+] antibodies are mutated, and because they are present almost uniquely in SLE serum, they offer the opportunity to study the pattern of somatic mutation in V_H genes used by lupus antibodies. While other SLE-associated idiotypes like 3I and 8.12 are expressed on protective antibodies in normal serum, F4[+] antibodies do not appear to be part of a protective response and are not present in normal serum. Therefore, we reasoned that the analysis of somatic mutation in F4[+] heavy chains might reveal a population of V_H genes displaying SLE-specific aspects of somatic mutation.

Analysis of R and S mutations displayed a clustering of R mutations in CDRs and a trend toward a higher than random R/S ratio in CDRs. These observations are more consistent with antigen selection than polyclonal activation, yet it is not possible from these studies to know whether the selecting antigen is a self or foreign antigen.

Smith and colleagues reported recently no difference in use of trinucleotide mutational hot spots between autoantibodies and anti–foreign antibodies in mice (32); however, they analyzed autoantibodies derived from MRL/lpr mice. MRL/lpr mice are autoimmune due to a defect in *fas* gene expression (40), which would not be expected to be involved in the process of somatic mutation. Our analysis reveals the same frequency of mutation in V_H genes encoding F4[+] heavy chains as in these genes encoding protective antibodies. However, we do find evidence for decreased targeting of somatic mutation to mutational hot spots when RGYW motifs are examined. When trinucleotide motifs are examined, there remains a targeting of somatic mutation to hot spots, but it is less than that seen in non-lupus heavy chains. This observation suggests that mutation can be uncoupled from targeting to hot spots. We would speculate that one complex is needed for mutation to occur, and that additional proteins are needed to target mutation to particular DNA sequences.

These results are similar to other studies in our laboratory which found that germinal center B cells of mice constitutively expressing bcl-2 show no decrease in frequency of somatic mutation of Ig genes, but a decreased targeting of mutation to putative mutational hot spots (41). Recent studies have shown that expression of bcl-2 inhibits progression through the cell cycle (42, 43). Possibly, the alteration in cell cycle kinetics leads to a change in the process of somatic mutation. More likely, constitutive expression of bcl-2 may alter gene expression in B cells and lead to an uncoupling of mutation from targeting to hot spots. Constitutive expression of bcl-2 or other alterations in B cell activation might affect expression of proteins needed to target mutation to hot spots. While the data remain inconclusive, it has been suggested that SLE B cells overexpress bcl-2 (44, 45).

It is possible, given the data presented, that there is a defect in the machinery of somatic mutation in SLE B cells, but it is more likely that the altered pattern of somatic mutation reflects a difference in B cell activation that may determine thresholds for negative selection. Alterations in B cell gene expression might affect both the targeting of somatic mutation and thresholds for negative selection. For example, increased expression of bcl-2, reported to occur in SLE B cells (44), affects both resistance to apoptosis and the targeting of somatic mutation to hot spot motifs. Alternatively, other changes in gene expression might lead to an uncoupling of mutation from targeting to hot spots. Because our analysis of mutation focuses exclusively on S mutations, we can conclude that the decreased frequency of mutation in hot spots is not a function of altered selection in SLE. We would speculate that the abnormal pattern of mutation is not itself responsible for the autospecificities that are present. Rather, we believe as stated above that overexpression of bcl-2, or some other change in B cell activation, is responsible for both the altered mutational pattern characterized by an uncoupling of mutation from targeting to hot spots, and the altered selection of B cells. We believe that SLE B cells may undergo germinal center maturation while in a different activation state. Taken together, these data might suggest that autospecificities in SLE arise due to a lack of negative selection of B cells acquiring autoreactivity through somatic mutation, rather than an antigen-specific activation of autoreactive cells. B cells in SLE may have an altered pattern of gene expression that leads to enhanced survival and an uncoupling of mutation from hot spot targeting.

Acknowledgments

References

1. Isenberg, D., W. Williams, J. Axford, R. Bakimer, D. Bell, T. Casaseca-Grayson, B. Diamond, F. Ebling, B. Hahn, G. Harkiss, et al. 1990. Comparison of DNA antibody idiotypes in human sera: an international collaborative study of 19 idiotypes from 11 different laboratories. *J. Autoimmun.* 3:393–414.

2. Diamond, B., and G. Solomon. 1983. A monoclonal antibody recognizes anti-DNA antibodies in patients with systemic lupus erythematosus. *Ann. NY Acad. Sci.* 418:379–385.

3. Davidson, A., A. Smith, J. Katz, J.L. Preud'Homme, A. Solomon, and B. Diamond. 1989. A cross-reactive idiotype on anti-DNA antibodies defines a H chain determinant present almost exclusively on IgG antibodies. *J. Immunol.* 143:174–180.

4. Grayzel, A., A. Solomon, C. Aranow, and B. Diamond. 1991. Antibodies elicited by pneumococcal antigens bear an anti-DNA–associated idiotype. *J. Clin. Invest.* 87:842–846.

5. Livneh, A., E. Gazit, and B. Diamond. 1994. The preferential expression of the anti-DNA associated 8.12 idiotype in lupus is not genetically controlled. *Autoimmunity.* 18:1–6.

6. Manheimer-Lory, A., J.B. Katz, M. Pillinger, C. Ghossein, A. Smith, and B. Diamond. 1991. Molecular characteristics of antibodies bearing an anti-DNA-associated idiotype. *J. Exp. Med.* 174:1639–1652.

7. Paul, E., A. Iliev, A. Livneh, and B. Diamond. 1992. The anti-DNA-associated idiotype 8.12 is encoded by the VλII gene family and maps to the vicinity of L chain CDR1. *J. Immunol.* 149:3588–3595.

8. Davidson, A., A. Manheimer-Lory, C. Aranow, R. Peterson, N. Hannigan, and B. Diamond. 1990. Molecular characterization of a somatically mutated anti-DNA antibody bearing two systemic lupus erythematosus–related idiotypes. *J. Clin. Invest.* 85:1401–1409.

9. Swanson, P.C., C. Ackroyd, and G.D. Glick. 1996. Ligand recognition by anti-DNA autoantibodies. Affinity, specificity and mode of binding. *Biochemistry.* 35:1624–1633.

10. Tomlinson, I.M., G. Walter, J.D. Marks, M.B. Llewelyn, and G. Winter. 1992. The repertoire of human germline V_H reveals about fifty groups of V_H segments with different hypervariable loops. *J. Mol. Biol.* 227:776–798.

11. Cox, J.P.L., I.M. Tomlinson, and G. Winter. 1994. A directory of human germline V_κ segments reveals a strong bias in their usage. *Eur. J. Immunol.* 24: 827–836.

12. Daley, M.D., H.-Q. Peng, V. Misener, X.-Y. Liu, P.P. Chen, and K. Siminovitch. 1992. Molecular analysis of human immunoglobulin Vλ germline genes: subgroups VλIII and VλIV. *Mol. Immunol.* 29:1515–1518.

13. Williams, C., and G. Winter. 1993. Cloning and sequencing of human immunoglobulin Vλ gene segments. *Eur. J. Immunol.* 23:1456–1461.

14. Radic, M.Z., J. Mackle, J. Erickson, C. Mol, W.F. Anderson, and M. Weigert. 1993. Residues that mediate DNA binding of autoimmune antibodies. *J. Immunol.* 150:4966–4977.

15. Seeman, N.C., J.M. Rosenberg, and A. Rich. 1976. Sequence-specific recognition of double helical nucleic acids by proteins. *Proc. Natl. Acad. Sci. USA.* 73:804–808.

16. O'Keefe, T.L., S.K. Datta, and T. Imanishi-Kari. 1992. Cationic residues in pathogenic anti-DNA autoantibodies arise by mutations of a germ-line gene that belongs to a large V_H gene subfamily. *Eur. J. Immunol.* 22:619–624.

17. Harada, T., N. Suzuki, Y. Mizushima, and T. Sakane. 1994. Usage of a novel class of germline Ig variable region gene for cationic anti-DNA autoantibodies in human lupus nephritis and its role for the development of the disease. *J. Immunol.* 153:4806–4815.

18. Demaison, C., P. Chastagner, J. Theze, and M. Zouali. 1994. Somatic diversification in the heavy chain variable region genes expressed by human autoantibodies bearing a lupus-associated nephritogenic anti-DNA idiotype. *Proc. Natl. Acad. Sci. USA.* 91:514–518.

19. Newkirk, M.M., H. Gram, G.F. Heinrich, L. Ostberg, J.D. Capra, and R.L. Wasserman. 1988. Complete protein sequences of the variable regions of the cloned heavy and light chains of a human anti-cytomegalovirus antibody reveal a striking similarity to human monoclonal rheumatoid factors of the Wa idiotypic family. *J. Clin. Invest.* 81:1511–1518.

20. Ikematsu, H., N. Harindranath, Y. Ueki, A.L. Notkins, and P. Casali. 1993. Clonal analysis of a human antibody response. II. Sequences of the V_H genes of human IgM, IgG and IgA to rabies virus reveal a preferential utilization of V_HIII segments and somatic hypermutation. *J. Immunol.* 150:1325–1337.

21. Harindranath, N., H. Ikematsu, A.L. Notkins, and P. Casali. 1993. Structure of the V_H and V_L segments of polyreactive and monoreactive human natural antibodies to HIV-1 and *Escherichia coli* β-galactosidase. *Int. Immunol.* 5:1523–1533.

22. Adderson, E.E., P.G. Shackelford, A. Quinn, P.M. Wilson, M.W. Cunningham, R.A. Insel, and W.L. Carroll. 1993. Restricted immunoglobulin VH usage and VDJ combinations in the human response to *Haemophilus influenzae* type b capsular polysaccharide. Nucleotide sequences of monospecific anti-*Haemophilus* antibodies and polyspecific antibodies cross-reacting with self antigens. *J. Clin. Invest.* 91:2734–2743.

23. Pirofski, L., R. Lui, M. DeShaw, A.B. Kressel, and Z. Zhong. 1995. Analysis of human monoclonal antibodies elicited by vaccination with a *Cryptococcus neoformans* glucoronoxylomannan capsular polysaccharide vaccine. *Infect. Immun.* 63:3005–3014.

24. Rioux, J.D., M. Ohlin, C.A.K. Borrebaeck, and M.M. Newkirk. 1995. Molecular characterization of human monoclonal antibodies specific for the human cytomegalovirus: relationship of variable region sequence to antigen specificity and rheumatoid factor-associated idiotype expression. *Immunol. Infect. Dis. (Lond.).* 5:43–52.

25. Hillson, J., N.S. Karr, I.R. Oppliger, M. Mannik, and E.H. Sasso. 1993. The structural basis of germline-encoded V_H3 immunoglobulin binding to staphylococcal protein A. *J. Exp. Med.* 178:331–336.

26. Rioux, J.D., J. Rauch, L. Silvestri, and M.M. Newkirk. 1994. A human rheumatoid factor C304 shares V_H and V_L gene usage with antibodies specific for human viral pathogens. *Scand. J. Immunol.* 40:350–354.

27. Pilkington, G.R., L. Duan, M. Zhu, W. Keil, and R.J. Pomerantz. 1996. Recombinant human Fab antibody fragments to HIV-1 Rev and Tat regulatory proteins: direct selection from a combinatorial phage display library. *Mol. Immunol.* 33:439–450.

28. Andris, J.S., P.H. Ehrlich, L. Ostberg, and J.D. Capra. 1992. Probing the human antibody repertoire to exogenous antigens. Characterization of the H and L chain V region gene segments from anti-hepatitis B virus antibodies. *J. Immunol.* 149:4053–4059.

29. Andris, J.S., S. Johnson, S. Zolla-Pazner, and J.D. Capra. 1991. Molecular characterization of five human anti-human immunodeficiency virus type I antibody heavy chains reveals extensive somatic mutation typical of an antigen-driven immune response. *Proc. Natl. Acad. Sci. USA.* 88:7783–7787.

30. Betz, A.G., M.S. Neuberger, and C. Milstein. 1993. Discriminating intrinsic and antigen-selected mutational hotspots in immunological V genes. *Immunol. Today.* 14:405–411.

31. Rogozin, I.B., and N.A. Kolchanov. 1992. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochem. Biophys. Acta.* 1171:11–18.

32. Smith, D.S., G. Creadon, P.K. Jena, J.P. Portanova, B.L. Kotzin, and L.J. Wysocki. 1996. Di- and trinucleotide target preferences of somatic mutagenesis in normal and autoreactive B cells. *J. Immunol.* 156:2642–2652.

33. Kieber-Emmons, T., J.M. von Feldt, A.P. Godillot, D. McCallus, V. Srikantan, D.B. Weiner, and W.V. Williams. 1994. Isolated V_H4+ heavy chain variable regions bind DNA. Characterization of a recombinant antibody heavy chain library derived from patient(s) with active SLE. *Lupus.* 3:379–392.

34. Brezinschek, H.P., R.I. Brezinschek, and P.E. Lipsky. 1995. Analysis of heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J. Immunol.* 155:190–202.

35. Manheimer-Lory, A., R. Monhian, A. Splaver, B. Gaynor, and B. Diamond. 1995. Analysis of the V_κI family: germline genes from an SLE patient and expressed autoantibodies. *Autoimmunity.* 20:259–265.

36. Bensimon, C., P. Chastagner, and M. Zouali. 1994. Human anti-DNA autoantibodies undergo essentially primary V_κ gene rearrangements. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2951–2962.

37. Suzuki, N., T. Harada, S. Mihara, and T. Sakane. 1996. Characterization of a germline V_κ gene encoding cationic anti-DNA antibody and role of receptor editing for development of the autoantibody in patients with systemic lupus erythematosus. *J. Clin. Invest.* 98:1843–1850.

38. Rioux, J.D., E. Zdarsky, M.M. Newkirk, and J. Rauch. 1995. Anti-DNA and anti-platelet specificities of SLE-derived autoantibodies: evidence for CDR2$_H$ mutations and CDR3 motifs. *Mol. Immunol.* 32:683–696.

39. Radic, M.Z., and M. Weigert. 1994. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu. Rev. Immunol.* 12:487–520.

40. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature.* 356:314–317.

41. Kuo, P., A. Alban, D. Gebhard, and B. Diamond. 1997. Overexpression of bcl-2 alters usage of mutational hot spots in germinal center B cells. *Mol. Immunol.* In press.

42. Mazel, S., D. Burtrum, and H.T. Petrie. 1996. Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. *J. Exp. Med.* 183:2219–2226.

43. O'Reilly, L.A., D.C.S. Huang, and A. Strasser. 1996. The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6979–6990.

44. Gatenby, P.A., and M. Irvine. 1994. The bcl-2 protooncogene is overexpressed in systemic lupus erythematosus. *J. Autoimmun.* 7:623–631.

45. Rose, L.M., D.S. Latchman, and D.A. Isenberg. 1995. Bcl-2 expression is unaltered in unfractionated peripheral blood mononuclear cells in patients with systemic lupus erythematosus. *Br. J. Rheumatol.* 34:316–320.