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V gene analysis of anti-cardiolipin antibodies from (NZW × BXSB) F_1 mice

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SUMMARY

In (NZW × BXSB) F_1 (W/B F1) male mice, systemic lupus-like disease, thrombocytopenia and coronary vascular disease with myocardial infarction occur, due to the presence of platelet-associated antibodies, anti-platelet antibodies and anti-cardiolipin antibodies (aCL). We developed monoclonal aCL and analysed the specificity of aCL. In the W/B F1 mice, there are aCL with pathogenic properties, which have an IgG isotype and reveal a cofactor-dependent binding to CL, binding activity to platelets, and lupus anti-coagulant (LA) activity. Here, we analysed the usage of V_H and Vκ genes of six aCL, including two pathogenic aCL, from W/B F1 mice, in an attempt to address the question of whether or not aCL with pathogenic properties use restricted Ig V genes. Sequence analysis of V_H and Vκ genes of aCL showed that the pathogenic aCL had V_HJ558 and Vκ21 or Vκ23 genes, whereas the other aCL without pathogenic features used mainly the 7183 V_H family and the random Vκ gene group. However, two pathogenic aCL showed a 86·6% homology with the IgV region, each other, indicating that they were not closely related clones. Thus, these findings suggest the possibility that usage of Ig V_H genes in pathogenic aCL is not random, but that there may exist a few epitopes of antigen recognized by the pathogenic aCL.

INTRODUCTION

Autoantibodies against cardiolipin (anti-cardiolipin antibodies; aCL) are often present in sera of patients with systemic lupus erythematosus (SLE) and related autoimmune disorders. These antibodies are apparently related to thromboembolic manifestations of cerebral or myocardial infarction, pulmonary embolism, deep venous thrombosis, intrauterine fetal loss due to placental infarction and thrombocytopenia.[1,2]

There is evidence that aCL cofactor (β_2-glycoprotein 1; β_2-gp1) is required for aCL to interact with cardiolipin (CL) and other anionic phospholipids,[3,4] and that aCL in SLE serum does not recognize β_2-gp1 or CL alone, yet does recognize the complex of CL and β_2-gp1 that appears after binding to CL, and anionic phospholipids.[3,5]

(NZW × BXSB) F_1 (W/B F1) male mice develop SLE-like manifestations, including several autoantibodies, circulating immune complexes and lupus nephritis. There is an abnormally high incidence of degenerative coronary vascular disease with myocardial infarction and thrombocytopenia, because of the presence of both platelet-associated antibodies and circulating anti-platelet antibodies in these animals.[6,7] In a recent study,[8] we demonstrated that W/B F1 male mice produced autoantibodies against CL; most of the aCL were the IgG type and the binding activity to CL was enhanced in the presence of mouse or human β_2-gp1. We developed monoclonal anti-cardiolipin antibodies from W/B F1 mice and clarified the specificity.[8]

In the present work, we analysed the usage of V_H and Vκ genes in hybridomas producing aCL from W/B F1 mice. The objective of the study was to determine the immunoglobulin V genes of the pathogenic aCL, which were of the IgG isotype and revealed a cofactor-dependent binding to CL, binding activity to platelets and lupus anti-coagulant (LA) activity, resulting in thrombocytopenia and thrombosis or infarction. We obtained evidence that pathogenic aCL uses the combination of the V_HJ558 and Vκ21 or Vκ23 genes, whereas other aCL represent mainly the 7183 V_H gene family and the random Vκ gene group.

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Abbreviations: aCL, anti-cardiolipin antibody; APTT, activated partial thromboplastin time; CDR, complementary determining region; CL, cardiolipin; DRVVT, dilute Russell's viper venom time; DTTI, dilute tissue thromboplastin inhibition test; β_2-gp1; β_2-glycoprotein 1; KCT, kaolin clotting time; LA, lupus anti-coagulant; M-BSA, methylated bovine serum albumin; PCR, polymerase chain reaction; RF, rheumatoid factor; W/B F1, (NZW × BXSB) F_1.

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MATERIALS AND METHODS

Mice and sera

W/B F1 male mice were purchased from Kiwa Laboratory Animals Co. Ltd, Wakayama, Japan. Sera from these animals were kept at −70° until use.

Table 1. Summary of sequence data of six aCL-producing hybridomas

Mouse	Clone	Isotype	V_H family*	D_H	J_H	Vκ group†	Jκ
1	W/B-CAL-1	IgG2a, κ	J558	Q52	4	21	2
1	W/B-CAL-2	IgM, κ	J558	SP-2.2, 3, 4, 5	4	Ox1	5
1	W/B-CAL-3	IgG2b, κ	J558	FL-16.2	2	23	5
2	W/B-CAL-4	IgM, κ	7183	Q52	3	10	1
2	W/B-CAL-5	IgM, κ	7183	Q52	3	10	1
2	W/B-CAL-6	IgG2a, κ	7183	SP-2.3, 4	3	23	5

*Nomenclature according to Brodeur and Riblet.[29]
†Nomenclature according to Potter *et al.*[30]
Homology searches were with GenBank.[23]

Cardiolipins and DNA

CL (Sigma Chemical Company, St Louis, MO) came supplied in ethanol. Micelles were formed in 0·01 M phosphate-buffered saline (PBS), pH 7·4, by sonication for 10 min at the concentration of 1 mg/ml. Calf thymus DNA was purchased from PL Biochemicals (Milwaukee, WI). Double-stranded (ds)DNA was obtained by the digestion of calf thymus DNA with S1 nuclease (Seikagaku Kogyo Co. Ltd, Tokyo, Japan), then dialysation and fractionation on a benzoyl-naphthoyl-DEAE–cellulose column (Serva Feinbiochemica, Heidelberg, Germany). Single-stranded (ss) DNA was prepared by heating dsDNA at 100° for 10 min, then immediately cooling in an ice bath.

β_2-gpI

Human aCL cofactor (β_2-gpI) was obtained using the following procedure. Sera from normal human subjects were chromatographed on a CL–polyacrylamide gel column. The elute was dialysed extensively against 14 mM sodium phosphate buffer, pH 7·4, and applied to a DEAE–cellulose column. To remove any contamination by IgG, the effluent from the column was further passed through a protein A–Sepharose column. The aCL cofactor proved to be β_2-gpI when the N-terminal amino acids were sequenced. Mouse β_2-gpI was also prepared from the serum pool of BALB/c mice, using the procedure described above.

Hybridomas

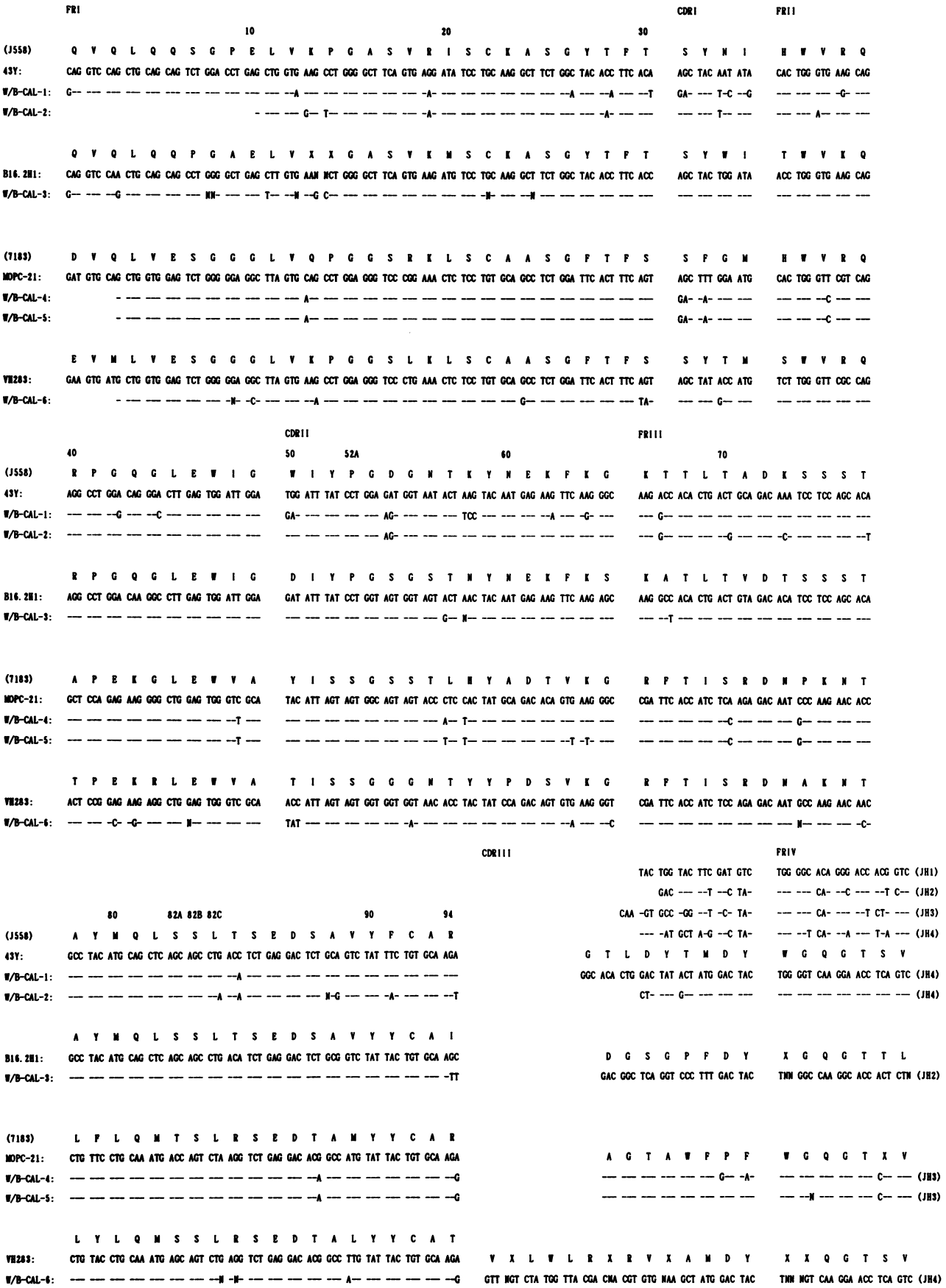
Six hybridomas producing aCL were made as described elsewhere.[8] Briefly, six monoclonal aCL were obtained by the fusion of 1×10^7 BALB/c myeloma line P3/X63-Ag8.U1 cells and 1×10^8 spleen cells from non-immunized W/B F1 male mice, producing high titres of aCL under the usual fusion protocol, as described elsewhere.[9] Monoclonal antibodies with cardiolipin-specific reactivity were screened by enzyme immunoassay (see below). The hybridomas were cloned by repeated procedures of limiting dilution and were maintained in RPMI-1640 enriched with 10% fetal calf serum (FCS). Ascites of monoclonal IgG aCL were purified using mAb Trap G Kits (Pharmacia LKB Biotechnology, Uppsala, Sweden). Monoclonal IgM aCL were obtained from the euglobulin fraction. The immunoglobulin isotypes of monoclonal antibodies were determined using a mouse monoclonal antibody isotyping kit (Amersham International plc, Amersham, U.K.).

Preparation of RNA and polymerase chain reaction (PCR)

Total RNA (100–200 μg) was prepared using RNA zol solution (Cinna/Biotecx Laboratories Inc., East Houston, TX) from $1–2 \times 10^7$ hybridoma cells producing aCL. Complementary DNA were synthesized from 5 μg total RNA in 20 μl reaction mixture containing oligo(dT) primer by avian myeloblastosis virus (AMV) reverse transcriptase. Amplification was performed with Taq polymerase in 50 μl of standard buffer using 0·2 μl of cDNA (corresponding to 50 ng of total RNA) with the following primers with a *Eco*RI cutting site. Primers specific for the V_HJ558 family: 5′-TCTAGAATTCGCAGGTGTCCACTCC-3′, positions -5--1, or 5′-TCTAGAATTCTCCGAGGTTCAGCTG-3′, positions -1-4; V_H7183 family, 5′-TCTAGAATTCGCTGGTGGAGTCTGG-3′, positions 4–8; V_H36-60 family, 5′-TCTAGAATTCGGGTATCCTGTCAGA-3′, positions -4-1; primers reactive for the Vκ5 group, 5′-TCTAGAATTCATAATGACCAGAGGA-3′, positions -5--1; Vκ8 group, 5′-TCTAGAATTCATTGTGATGACACAG-3′, positions 2–6; Vκ12-13 group, 5′-TCTAGAATTCGCCAGATGTGACATC-3′, positions -3-2; Vκ21 group, 5′-TCTAGAATTCTCCACAGGTGACATT-3′, positions -3-2; Vκ22 group, 5′-TCTAGAATTCGGAGACATTGTGATG-3′, positions -1-4; Vκ23 group, 5′-TCTAGAATTCAGCCTCCAGAGGTGA-3′, positions -4-1; primer for Cγ (5′-TCTAGAATTCGGCCAGTGGATAGA-3′, positions 121–125; Cμ (5′-TCTAGAATTCGCTCTCGCAGGAGA-3′, positions 125–129; Cκ (5′-TCTAGAATTCTGCAGCATCAGCCC-3′, positions 108–112). Oligonucleotides were synthesized in a 380A DNA synthesizer. Denaturing was done at 94° for 1·5 min, annealing at 60° for 1 min, and extension at 72° for 1 min, for 30 cycles on a DNA Thermal Cycler (Zymoreactor V2, Atto Co. Ltd, Tokyo, Japan). PCR products were separated on a 2% agarose gel, and amplified DNA bands encoding $V_HDJ_HC_H$ or VκJκCκ genes were examined. PCR products yielded about 400 base pairs.

Cloning and sequencing of cDNAs encoding V_H and Vκ genes

PCR products were purified by phenol-chloroform-isoamylalcohol extraction and precipitated with ethanol, followed by restriction enzyme digestion in excess amounts of *Eco*RI. Fragments of expected sizes for the cDNAs were enriched by preparative 2% low melting agarose gel (BRL, Gaithersburg, MD) electrophoresis. The DNA was again extracted with phenol-chloroform-isoamylalcohol, precipitated with ethanol, and resuspended in double-distilled water.


FR1
CDR1
FR11
10
20
30
(J558) Q V Q L Q Q S G P E L V K P G A S V R I S C K A S G Y T F T S Y N I H W V R Q
43Y: CAG GTC CAG CTG CAG CAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCT TCA GTG AGG ATA TCC TGC AAG GCT TCT GGC TAC ACC TTC ACA AGC TAC AAT ATA CAC TGG GTG AAG CAG
W/B-CAL-1: G-- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- -A- --- --- --- --- --- --- --A --- --A --- --T GA- --- T-C --G --- --- --- -G- ---
W/B-CAL-2: - --- --- G-- T-- --- --- --- --- -A- --- --- --- --- --- --- --- --- -A- --- --- --- T-- --- --- --- A-- --- ---
Q V Q L Q Q P G A E L V X X G A S V K M S C K A S G Y T F T S Y W I T W V K Q
B16.2H1: CAG GTC CAA CTG CAG CAG CCT GGG GCT GAG CTT GTG AAN MCT GGG GCT TCA GTG AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACC AGC TAC TGG ATA ACC TGG GTG AAG CAG
W/B-CAL-3: G-- --- --G --- --- --- --- NN- --- --- T-- --N --G C-- --- --- --- --- --- --- --- -M- --- --N --- --- --- --- --- --- --- --- --- --- --- --- --- ---
(7183) D V Q L V E S G G G L V Q P G G S R K L S C A A S G F T F S S F G M H W V R Q
MDPC-21: GAT GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA GGG TCC CGG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TTT GGA ATG CAC TGG GTT CGT CAG
W/B-CAL-4: - --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- GA- -A- --- --- --- --- --C --- ---
W/B-CAL-5: - --- --- --- --- --- --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- GA- -A- --- --- --- --- --C --- ---
E V M L V E S G G G L V K P G G S L K L S C A A S G F T F S S Y T M S W V R Q
VH2B3: GAA GTG ATG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG TCT TGG GTT CGC CAG
W/B-CAL-6: - --- --- --- --- --- -N- -C- --- --- --A --- --- --- --- --- --- --- --- --- G-- --- --- --- --- --- TA- --- --- G-- --- --- --- --- --- --- ---
CDR11
FR111
40
50
52A
60
70
(J558) R P G Q G L E W I G W I Y P G D G N T K Y N E K F K G K T T L T A D K S S S T
43Y: AGG CCT GGA CAG GGA CTT GAG TGG ATT GGA TGG ATT TAT CCT GGA GAT GGT AAT ACT AAG TAC AAT GAG AAG TTC AAG GGC AAG ACC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA
W/B-CAL-1: --- --- --G --- --C --- --- --- --- --- GA- --- --- --- --- AG- --- --- --- TCC --- --- --- --A --- -G- --- --- G-- --- --- --- --- --- --- --- --- ---
W/B-CAL-2: --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- AG- --- --- --- --- --- --- --- --- --- --- --- G-- --- --- --G --- --- -C- --- --- --- --T
R P G Q G L E W I G D I Y P G S G S T N Y N E K F K S K A T L T V D T S S S T
B16.2H1: AGG CCT GGA CAA GGC CTT GAG TGG ATT GGA GAT ATT TAT CCT GGT AGT GGT AGT ACT AAC TAC AAT GAG AAG TTC AAG AGC AAG GCC ACA CTG ACT GTA GAC ACA TCC TCC AGC ACA
W/B-CAL-3: --- G-- N-- --- --- --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---
(7183) A P E K G L E W V A Y I S S G S S T L H Y A D T V K G R F T I S R D N P K N T
MDPC-21: GCT CCA GAG AAG GGG CTG GAG TGG GTC GCA TAC ATT AGT AGT GGC AGT AGT ACC CTC CAC TAT GCA GAC ACA GTG AAG GGC CGA TTC ACC ATC TCA AGA GAC AAT CCC AAG AAC ACC
W/B-CAL-4: --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- T-- --- --- --- --- --- --- --- --C --- --- --- G-- --- --- ---
W/B-CAL-5: --- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- T-- T-- --- --- --- --- --T -T- --- --- --- --- --C --- --- --- G-- --- --- ---
T P E K R L E W V A T I S S G G G N T Y Y P D S V K G R F T I S R D N A K N T
VH2B3: ACT CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT GGT AAC ACC TAC TAT CCA GAC AGT GTG AAG GGT CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC AAC
W/B-CAL-6: --- --- -C- -G- --- --- N-- --- --- --- TAT --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --A --- --C --- --- --- --- --- --- --- --- --- N-- --- --- -C-
CDR111
FR1V
80
82A 82B 82C
90
94
(J558) A Y M Q L S S L T S E D S A V Y F C A R
43Y: GCC TAC ATG CAG CTC AGC AGC CTG ACC TCT GAG GAC TCT GCA GTC TAT TTC TGT GCA AGA G T L D Y T M D Y W G Q G T S V
W/B-CAL-1: --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- GGC ACA CTG GAC TAT ACT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC (JH4)
W/B-CAL-2: --- --- --- --- --- --- --- --A --A --- --- --- --- N-G --- --- -A- --- --- --T CT- --- G-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- (JH4)
A Y M Q L S S L T S E D S A V Y Y C A I
B16.2H1: GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA AGC D G S G P F D Y X G Q G T T L
W/B-CAL-3: --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --TT GAC GGC TCA GGT CCC TTT GAC TAC TNN GGC CAA GGC ACC ACT CTN (JH2)
(7183) L P L Q M T S L R S E D T A M Y Y C A R
MDPC-21: CTG TTC CTG CAA ATG ACC AGT CTA AGG TCT GAG GAC ACG GCC ATG TAT TAC TGT GCA AGA A G T A W F P F W G Q G T X V
W/B-CAL-4: --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --G --- --- --- --- --- --- G-- -A- --- --- --- --- --- C-- --- (JH3)
W/B-CAL-5: --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --G --- --- --- --- --- --- --- --- --- --N --- --- --- --- C-- --- (JH3)
L Y L Q M S S L R S E D T A L Y Y C A T
VH2B3: CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC TTG TAT TAC TGT GCA AGA V X L W L R X R V X A M D Y X X Q G T S V
W/B-CAL-6: --- --- --- --- --- --- --- --N -N- --- --- --- --- --- A-- --- --- --- --- --G GTT NGT CTA TGG TTA CGA CNA CGT GTG NAA GCT ATG GAC TAC TNN NGT CAA GGA ACC TCA GTC (JH4)

These DNA fragments were cloned into the *Eco*RI site of M13mp19 plasmids. The ligated DNA was introduced into *Escherichia coli*, TG-1 strain, under the standard transformation procedure described by Hanahan.[10] A single plaque was picked up and single-stranded DNA was purified for DNA sequence determination. Sequencing of specific clones was done by automated sequencing (Applied Biosystems, Co. Ltd, Foster City, CA). Homology searches were made using GenBank and EMBL libraries.[23]

Enzyme-linked immunosorbent assay (ELISA) to CL

Binding activity to CL was measured by solid-phase ELISA as described elsewhere.[11] Briefly, each well of polyvinylchloride microtitre plates (Dynatec Laboratories, Alexandria, VA) was coated with 50 μl of CL (50 μg/μl) in ethanol, and ethanol was evaporated under air at room temperature. Each well was incubated with 200 μl of 10% FCS for 1 hr at room temperature. After washing with PBS containing 0·05% Tween 20 (PBS–Tween), the plates were incubated with hybridoma supernates (50 μl) or purified monoclonal antibodies for 1 hr at room temperature. These were then extensively washed and reacted with alkaline phosphatase-labelled goat anti-mouse IgG plus IgM (Tago Inc., Burlingame, CA) for another 1 hr at room temperature. The plates were again washed before the addition of 0·1% p-nitrophenyl phosphate disodium in 1 M diethanolamine buffer, pH 9·8. After a 1 hr incubation at room temperature, OD was measured at 405 nm using a Corna Microplate Reader, MTP-100 (Corona Electric Co. Ltd, Ibaragi, Japan). The concentration of antibodies bound to CL was proportional to colour change of the substrate, measured as the absorbance at 405 nm.

Cofactor-dependent binding of monoclonal aCL

The CL-coated wells were incubated for 10 min with 50 μl of the purified mouse or human β_2-gp1, which were sequentially diluted (0–10·0 μg/ml), then 50 μl of monoclonal aCL was added and the preparation incubated for 1 hr at room temperature. After washing in PBS, the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG plus IgM antibody for 1 hr at room temperature. p-Nitrophenyl phosphate was added to each well and the OD was measured at 405 nm.

ELISA to DNA

Antibodies to DNA were measured by a solid-phase ELISA, as described elsewhere.[11] Each well was first coated with 100 μl of 0·1% methylated bovine serum albumin (M-BSA) solution for 1 hr at room temperature, and then incubated with 50 μl of DNA (10 μg/ml) in PBS containing 1 mM EDTA and 0·05% Tween 20 (PBS–EDTA–Tween) for 1 hr at room temperature, after discarding the M-BSA. Plates were incubated with 50 μl of monoclonal antibodies (10 μg/ml) for 1 hr at room temperature then were extensively washed in PBS and reacted with alkaline phosphatase-labelled goat anti-mouse IgG plus IgM antibodies. After a 1 hr incubation, the plates were again washed before the addition of enzyme substrate and the OD was measured at 405 nm, using a Corona Microplate Reader.

Flow cytometry

Binding activity of aCL to platelets was analysed by flow cytometry, as described elsewhere.[7] The platelets from BALB/c mice were suspended in 1% paraformaldehyde solution for 5 min. After washing twice in PBS, the platelets were resuspended in EDTA–PBS to a platelet count of 2×10^6/100 μl. To detect the antibodies, aliquots of platelets were incubated with 100 μl of monoclonal aCL (10 and 50 μg/ml) for 30 min at room temperature. After washing twice in EDTA–PBS, they were then incubated with FITC-conjugated antisera (goat anti-mouse Ig; Cappel Laboratories, West Chester, PA) for 30 min at room temperature. After a final washing, samples were then analysed on a FACScan analyser (Becton Dickinson, Mountain View, CA) by gating to exclude debris.

Anti-coagulant activity of aCL

Anti-coagulant activity of aCL was determined by elongation of an activated partial thromboplastin time (APTT), kaolin clotting time (KCT), modified dilute Russell's viper venom time (DRVVT) and dilute tissue thromboplastin inhibition test (DTTI). APTT, DRVVT and KCT tests were carried out as described previously,[12] as was DTTI.[13]

RESULTS

Usage of V_H genes in aCL from W/B F1 mice

The V_H and Vκ regions of six monoclonal aCL from two W/B F1 mice were sequenced. Sequence data of six hybridomas producing aCL showed that aCL were encoded by two V_H families (Table 1). The J558 V_H genes were preferentially used in three (W/B-CAL-1, 2 and 3) of six aCL, and the other three clones (W/B-CAL-4, 5 and 6) used the 7183 V_H gene. As shown in Fig. 1, sequences in a single J558 V_H gene were separated into two groups. The genes were compared to known genes of the J558 V_H family, 43Y for group 1 and B16.2H1 for group 2, as described by Rathbun *et al.*[14] and by Förster *et al.*,[15] respectively. Two clones (W/B-CAL-1 and 2) shared a 87·9% homology and showed a 91·5% and 92·8% homology to 43Y, respectively. The V_H gene of W/B-CAL-3 clone had a 86·6% homology to the W/B-CAL-1 gene and a 95·9% homology to B16.2H1. Sequences of the 7183 V_H gene were separated into two groups and they were compared to known genes of the 7183 V_H family, the MOPC-21 gene[16] and VH283 gene.[17] W/B-CAL-4 and 5 clones shared a 99·0% homology and were closely related. They had a 96·1% and 95·4% homology to MOPC-21, respectively. W/B-CAL-6 clone had only 84·5–85·9% homology to the W/B-CAL-4 and 5 clones and showed a 92·3% homology to V_H283.

Usage of Vκ genes in aCL from W/B F1 mice

Sequence data of aCL showed that these were encoded by four different Vκ gene groups (Table 1). The Vκ10 and Vκ23 gene groups were used in two out of six hybridomas, respectively. The other two clones belonging to VκOx1 and Vκ21 gene groups. Figure 2 shows that sequences in the Vκ10 gene group

Figure 1. V_H region sequences of the six hybridomas. V gene sequences were compared to the 43Y,[14] B16.2H1,[15] MOPC-21[16] and VH283[17] genes. 43Y is a germ-line gene from an A/J mouse. B16.2H1 is a re-arranged gene from a CB.20 mouse. MOPC-21 is a re-arranged gene from C57Bl/6 mouse. V_H283 is a germ-line gene from a BALB/c mouse.

```
            FRI                                                                                CDRI
                                      10                        20                                          27A 27B 27C 27D     30
(VKOX1)     Q   I   V   L   T   Q   S   P   A   I   M   S   A   S   P   G   E   K   V   T   M   T   C     S   A   S                     S   S   V   S   Y   M   H
45.21.1:    CAA ATT GTT CTC ACC CAG TCT CCA GCA ATC ATG TCT GCA TCT CCA GGG GAG AAG GTC ACC ATG ACC TGC   AGT GCC AGC                   TCA AGT GTA AGT TAC ATG CAC
W/B-CAL-2:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- ---                   --- --- --- --- --- --- ---

(VK10)      D   I   Q   M   T   Q   T   T   S   S   L   S   A   S   L   G   D   R   V   T   I   S   C     R   A   S   Q                 D   I   S   N   Y   L   N
VK-GL:      GAT ATC CAG ATG ACA CAG ACT ACA TCC TCC CTG TCT GCC TCT CTG GGA GAC AGA GTC ACC ATC AGT TGC   AGG GCA AGT CAG               GAC ATT AGC AAT TAT TTA AAC
W/B-CAL-4:  --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- ---               --- --- --- --- --- --- ---
W/B-CAL-5:  --C --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- ---               --- --- --- --- --- --- ---

(VK21)      D   I   V   L   T   Q   S   P   A   S   L   A   V   S   L   G   Q   R   A   T   I   S   C     R   A   S   K   S   V   S   T   S   G   Y   S   Y   M   H
GHP53:      GAC ATT GTG CTG ACA CAG TCT CCT GCT TCC TTA GCT GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TGC   AGG GCC AGC AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC
W/B-CAL-1:  --- --- -A- --- --- --- --- --- -T- --- N-- -T- --- --- --- --- --- --- -T- --- --- --- ---   --- --- --- --- --- --- --- --- -T- --- N-- --- --- --- ---

(VK23)      D   I   V   L   T   Q   S   P   A   T   L   S   V   T   P   G   D   S   V   S   L   S   C     R   A   S   Q                 S   I   S   N   N   L   H
126:        GAT ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT GTG ACT CCA GGA GAT AGC GTC AGT CTT TCC TGC   AGG GCC AGC CAA               AGT ATT AGC AAC AAC CTA CAC
W/B-CAL-3:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --C ---               --- --- --- --- --- --- ---
W/B-CAL-6:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- ---               --- --- --- --- --- --T ---

                                                                CDRII                         FRIII
                37          40                                  50                                                60                                      70
(VKOX1)     W   Y   Q   Q   K   S   G   T   S   P   K   R   W   I   Y     G   T   S   K   L   A   S     G   V   P   A   R   F   S   G   S   G   S   G   T   S   Y   S   L
45.21.1:    TGG TAC CAG CAG AAG TCA GGC ACC TCC CCC AAA AGA TGG ATT TAT   GAC ACA TCC AAA CTG GCT TCT   GGA GTC CCT GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC
W/B-CAL-2:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   -G- --- --- --- --- --- ---   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C-

(VK10)      W   Y   Q   Q   K   P   D   G   T   V   K   L   L   I   Y     Y   T   S   R   L   H   S     G   V   P   S   R   F   S   G   S   G   S   G   T   D   Y   S   L
VK-GL:      TGG TAT CAG CAG AAA CCA GAT GGA ACT GTT AAA CTC CTG ATC TAC   TAC ACA TCA AGA TTA CAC TCA   GGA GTC CCA TCA AGG TTC AGT GGC AGT GGG TCT GGA ACA GAT TAT TCT CTC
W/B-CAL-4:  --- --- --- --- --- --- --- --- --- --- --G --- --- --- ---   --- --- --- --- --- --- ---   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- N--
W/B-CAL-5:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --- ---   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

(VK21)      W   T   Q   Q   K   P   G   Q   P   P   K   L   L   I   Y     L   A   S   N   L   E   S     G   V   P   A   R   F   S   G   S   G   S   G   T   D   F   T   L
GHP53:      TGG TAC CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT   CTT GCA TCC AAC CTA GAA TCT   GGG GTC CCT GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC
W/B-CAL-1:  --- -G- --- --- TT- --- -TT --- --- --- --- --- --- --- ---   -N- --- --- --- --- --- ---   --- --- --C --- --- --- --- --- --- --- --- --- T-- --- --- --- ---

(VK23)      W   Y   Q   Q   K   S   H   E   S   P   R   L   L   I   K     Y   A   S   Q   S   I   S     G   I   P   S   R   F   S   G   S   G   S   G   T   D   F   T   L
126:        TGG TAT CAA CAA AAA TCA CAT GAG TCT CCA AGG CTT CTC ATC AAG   TAT GCT TCC CAG TCC ATC TCT   GGG ATC CCC TCC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACT CTC
W/B-CAL-3:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --- ---   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -N- AG-
W/B-CAL-6:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   -T- A-- --- --- --- --- ---   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- N--

                                                                CDRIII                              FRIV
                                                                                        TGG ACG     TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA (JK1)
                                                                                        -AC ---     --- --A --G --G --- --- --- --- --A --- (JK2)
                                                                                        ATC --A     --- A-- -AT --G --- -GA --- --- --A --- (JK3)
                                                                                        -TC ---     --- --C TCG --G --A --- T-- --- --A --- (JK4)
                74                      80                          90                   95 CTC ---     --- --- -CT --G --- --- --- --G C-G --- (JK5)
(VKOX1)     T   I   S   S   M   E   A   E   D   A   A   T   Y   Y   C     Q   Q   W   S   S   N   P
45.21.1:    ACA ATC AGC AGC ATG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC   CAG CAG TGG AGT AGT AAC CCA  L   T       F   G   A   G   T   K   L   E   L   K
W/B-CAL-2:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --- --C CTC ACG     TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA (JK5)

(VK10)      T   I   S   N   L   E   Q   E   D   I   A   T   Y   F   C     Q   Q   G   N   T   L   P
VK-GL:      ACC ATT AGC AAC CTG GAG CAA GAA GAT ATT GCC ACT TAC TTT TGC   CAA CAG GGT AAT ACG CTT CCT  W   T       F   G   G   G   T   K   L   E   I   K
W/B-CAL-4:  --- --- --- --- --- --- --- --- -G- --- --- --- --- --- ---   --- --- --- --- --- --- --G TGG ACG     TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA (JK1)
W/B-CAL-5:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --- --G --- ---     --- --- --- --- --- --- --- --- --- --- (JK1)

(VK21)      N   I   H   P   V   E   E   E   D   A   A   T   Y   Y   C     Q   H   S   R   E   L   P
GHP53:      AAC ATC CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT   CAG CAC AGT AGG GAG CTT CCT  Y   T       F   G   G   G   T   K   L   E   V   K
W/B-CAL-1:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --C --G TAC ACG     TTC GGA GGG GGG ACC AAG CTG GAA GTA AAA (JK2)

(VK23)      S   I   N   S   V   E   T   E   D   F   G   M   Y   F   C     Q   Q   S   N   S   W   P
126:        AGT ATC AAC AGT GTG GAG ACT GAA GAT TTT GGA ATG TAT TTC TGT   CAA CAG AGT AAC AGC TGG CCT  L   T       F   G   A   G   T   K   L   E   L   K
W/B-CAL-3:  --- --- --- --- --- --- --- --- --- --- --- G-- -C- --- ---   --- --- --- --- --- --- --- CTC ACG     TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA (JK5)
W/B-CAL-6:  --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   --- --- --- --- --- --- --- --- ---     --- --- --- --- --- --- --- --- --- --- (JK5)
```

Table 2. Properties of aCL and sequences of V_H CDR3 region

Mouse	Clone	Binding activity to				LA activity	Cofactor dependency		V_H CDR3
		CL	ssDNA	dsDNA	Plt		Mouse	Human	
1	W/B-CAL-1	+	−	−	+	+	+	+	gtldytmdy
1	W/B-CAL-2	+	+	+	−	−	−	−	lyamdy
1	W/B-CAL-3	+	−	−	+	−	+	+	dgsgpfdy
2	W/B-CAL-4	+	−	−	−	ND	−	−	agtawfay
2	W/B-CAL-5	+	−	−	−	ND	−	−	agtawfpf
2	W/B-CAL-6	+	+	−	−	ND	−	−	vxlwl**RxR**vxamdy

Binding activities to CL, ss/dsDNA, platelets (Plt) and LA activities, mouse or human cofactor-dependent binding of aCL, and sequences of V_H CDR3 region. Binding activity to CL, ss/dsDNA and platelets was assayed according to the procedure outlined in the Materials and Methods, as was determination of LA activities of mAb.

+, positive activity; −, negative activity; ND, not determined.

In the V_H CDR3 column is given the single letter amino acid sequences of the V_H CDR3 region.

R, arginine residues.

can be compared to the known members of the Vκ10 gene group, VK-GL.[18] Two clones (W/B-CAL-4 and 5) shared a 98% homology to VK-GL and were closely related. The other two clones (W/B-CAL-3 and 6) could be compared to the known Vκ23 gene, 126.[19] They were 98% homologous to 126 and were related. The other Vκ genes could be compared to the known genes of VκOx1, 45.21.1,[20] and Vκ21, GHP53.[21]

Usage of J_H, D and Jκ genes

Three J_H and Jκ genes were found in six hybridomas and there was no particular restriction (Table 1). The related clones (W/B-CAL-4 and 5) from one mouse used the same D region.

Binding activities to CL, ss/dsDNA, platelets and anti-coagulant (LA) activities of aCL

Binding activity to CL, ss/dsDNA and platelets was detected by solid-phase ELISA and flow cytometry, respectively, as described in the Materials and Methods. The aCL from all hybridomas had binding activities to CL (Table 2). To determine the cofactor dependency of monoclonal aCL, we carried out ELISA using purified human or mouse β_2-gpl, as described in the Materials and Methods. As shown in Fig. 3, the CL binding activity of W/B-CAL-1 and 3 was elevated proportionately by the additions of sequentially diluted purified human or mouse β_2-gpl. The other four aCL (W/B-CAL-2, 4, 5 and 6) showed no β_2-gpl-dependent CL binding. Two aCL (W/B-CAL-2 and 6) could also bind to ssDNA but only W/B-CAL-2 bound to both ssDNA and dsDNA. Two hybridomas (W/B-CAL-1 and 3) revealed binding activity to platelets, but the other four clones did not.

LA activities of aCL were carried out as described in the Materials and Methods. Table 2 shows that only W/B-CAL-1 had activity to prolong the clotting in coagulation *in vitro*. The

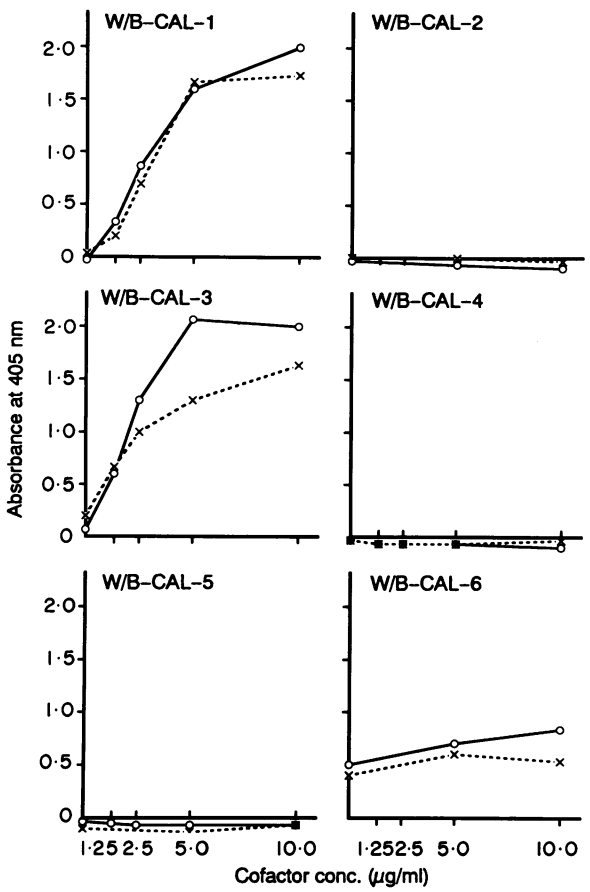


Figure 3. Cofactor-dependent binding of monoclonal aCL. The aCL (W/B-CAL-1, 2, 3, 4, 5 and 6) were incubated with CL-coated wells pretreated with sequentially diluted (0–10·0 μg/ml) mouse (×) or human (○) β_2-gpl. The binding activity of aCL was measured as the OD at 405 nm.

Figure 2. Vκ region sequences of the six hybridomas. Vκ region sequences were compared to the 45.21.1,[20] VK-GL,[18] GHP53[21] and 126[19] genes. 45.21.1 is a re-arranged gene from a C57BL/6JA mouse. VK-GL is a germ-line gene from a A/J mouse. GHP53 is a germ-line gene from a BALB/c mouse. 126 is a re-arranged gene from a BALB/c mouse.

W/B-CAL-1, which represented the binding activity to platelets, LA activity and cofactor-dependency binding to CL, used the J558 V_H gene family and the Vκ21 gene group.

Amino acid sequences of the aCL V_H complementary determining (CDR) 3 region

Amino acid sequencing of the aCL V_H CDR3 region was carried out to examine the relationship between the binding specificity and amino acid substitutions in the V_H CDR3 region. Table 2 shows that only W/B-CAL-6 had two arginines in CDR3 of its H chain, and the other five clones contained no arginine residues. W/B-CAL-6 revealed binding activity to ssDNA, but W/B-CAL-2 binding activity to ssDNA and dsDNA represented no arginine in the V_H CDR3 region.

DISCUSSION

Pathogenic aCL represent the IgG isotype, cofactor-dependent binding to CL, binding activity to platelets and LA activity.[4] Our studies on the specificity of aCL using monoclonal aCL revealed that aCL exist with pathogenic properties in W/B F1 mice. To determine whether or not, pathogenic aCL use the restricted Ig V genes, we examined the usage of V_H and Vκ region sequences of six aCL, including two pathogenic aCL (W/B-CAL-1 and 3). These two aCL induced thrombosis *in vivo* by injection of monoclonal antibodies (mAb) into mice (data not shown). Sequence analysis of V_H and Vκ genes of aCL showed that the pathogenic aCL had V_HJ558 and Vκ21 or Vκ23 genes, while the other aCL without complete pathogenic features used mainly the 7183 V_H gene family and the random Vκ gene group. However, W/B-CAL-1 had a 86·6% homology to W/B-CAL-3 although they were thought not to be closely related clones. Furthermore, there were no restricted D_H and J_H genes among these two pathogenic aCL. These findings suggest the possibility that usage of Ig V_H genes in the pathogenic aCL might be limited and there may exist a few epitopes of antigen recognized by the pathogenic aCL.

Shlomchik *et al.*[22] analysed the gene usage of rheumatoid factors (RF) and anti-DNA mAb from MRL-lpr/lpr mice and clarified that the B-cell producing RF and anti-DNA mAb were oligoclonal. We previously examined the V_H/Vκ region sequences of 14 monoclonal aCL from MRL-lpr/lpr mice and clarified that B cells producing aCL are also oligoclonal.[23] These findings indicate that RF, anti-DNA mAb and aCL in MRL-lpr/lpr mice might be generated from a limited B-cell precursor. In contrast, in the present work, we analysed Ig V gene of two aCL with pathogenic properties in W/B F1 mice but we could not elucidate whether these aCL were derived from oligoclonal B cells or not.

Analyses on nucleotide sequences of the V region of anti-DNA mAb from MRL-lpr/lpr mice showed that somatic mutations could cause a specificity for ss/dsDNA and that arginine residues in the V_H CDR regions might play an important role.[22] Our studies show that W/B-CAL-6, with binding activity to ssDNA, represents two arginine residues in the V_H CDR3 region, but W/B-CAL-2, with binding activity to both ssDNA and dsDNA, does not contain an arginine residue. Thus, these data suggest that arginine residues in the V_H CDR3 region of aCL from W/B F1 mice may not always be significant for the specificity to DNA. Arginine residues in the V_H CDR3 region do not correlate with the binding activity to platelets, LA activity and cofactor-dependent binding to CL, since there is no arginine in the V_H CDR3 of pathogenic aCL.

The antibodies from secondary or hyperimmune responses have an increased affinity for immunizing antigen, compared to typical primary response antibodies. This correlates with the presence of somatic mutations in the V regions of secondary antibodies in various antigenic systems.[24] Several investigations[22,25,26] showed that IgG anti-DNA antibodies are encoded by V genes that have diverged from germ-line gene segments through somatic mutation, in a manner of antigen-driven B-cell activation in lupus-prone mice and in SLE patients. Taki *et al.*[27] compared sequences of the V_H gene encoding IgG or IgM anti-DNA antibody with the related germ-line V_H gene; the V_H sequence of the IgG anti-DNA antibody, which showed a higher DNA binding activity than did the IgM antibody, contained somatically mutated nucleotides. They speculated that the germ-line encoded, low-affinity IgM autoantibody undergoes somatic mutations and an isotype switch, resulting in the generation of pathogenic, high-affinity autoantibodies. van Es *et al.*[28] found that the V_H gene encoding a monoclonal human IgG aCL (R149) from a patient with active SLE was a somatically mutated variant of the germ-line gene segment. Our previous studies[23] showed that three to five somatic mutations in the V_H region were present in monoclonal aCL from MRL-lpr/lpr mice compared with the related germ-line V_H gene. In this study, the pathogenic aCL (W/B-CAL-1) shared a 91·5% homology to the known germ-line V_H gene, 43Y, suggesting that this aCL clone with the pathogenic feature was generated by somatic mutations. However, there is no evidence that the 43Y gene is the origin of the W/B-CAL-1 V_H gene. A comparison of the V_H gene encoding pathogenic aCL with the genetically related germ-line V_H gene will shed light on mechanisms underlying these events in subjects with autoimmune disease.

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