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Characterization of a human monoclonal autoantibody directed to cardiolipin/β_2 glycoprotein I produced by chronic lymphocytic leukaemia B cells

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SUMMARY

We determined the specificity and sequence of immunoglobulin molecules synthesized by monoclonal B cells from a patient with chronic lymphocytic leukaemia (CLL) who presented with a number of clinical and biological autoimmune symptoms. Heterohybrids obtained by fusion of CLL cells with the mouse X63-Ag 8.653 myeloma produced IgMλ MoAbs directed to the cardiolipin/β_2 glycoprotein I (β2GPI) complex and ssDNA. They were devoid of polyreactivity. Nucleotide sequence analysis of the variable domain of the μ chain indicated the utilization of the V_H4 71.2 gene or one allotypic variant, $D_{XP}4$ and J_H3 segments. The λ light chain used the single gene from the $V_\lambda8$ subfamily, $J_\lambda3$ and $C_\lambda3$ genes. The V_H gene displayed 11 nucleotide changes in comparison with its putative germline counterpart. However, these nucleotide changes correspond to variations observed in other published V_H4 sequences, suggesting gene polymorphism rather than somatic mutation. $D_{XP}4$ and J_H3 were also in germline configuration. The V_L gene exhibited a single replacement mutation in CDR1. These data suggest that the monoclonal CLL B cells in this patient retained V_H and V_L genes in germline configuration although they secreted a pathogenic anti-cardiolipin antibody associated with clinical symptoms, vasculitis and thrombosis, which may be provoked by antibodies to the phospholipid/β2GPI complex.

Keywords anti-cardiolipin/β_2 glycoprotein I monoclonal antibody nucleotide sequence B chronic lymphocytic leukaemia

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is a clonal B cell malignancy expressing the CD5 antigen. These leukaemic cells, as well as the normal CD5 B cells, often produce natural autoantibodies [1–3]. Typically, the latter are low affinity polyspecific antibodies, encoded by a restricted number of germline V_H and V_L genes [4,5]. Usually, these antibodies are not responsible for clinical manifestations; in contrast, pathogenic autoantibodies frequently observed in CLL patients are not produced by the malignant clone.

We studied a patient with CLL who presented with a constellation of clinical and biological autoimmune symptoms; to investigate whether in this particular setting CLL cells could produce pathogenic autoantibodies, we determined the antibody activity of the monoclonal IgM secreted by CLL cells as well as its nucleotide sequence.

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PATIENT, MATERIALS AND METHODS

Patient

This 58-year-old patient presented with a $CD5^+$ B CLL featuring $\mu\lambda$ membrane immunoglobulin. The patient suffered several months of fever, arthralgias and Raynaud's phenomenon. A sural thrombophlebitis occurred without underlying cause. Clinical examination revealed a splenomegaly, superficial lymph nodes and an urticarial skin rash. Skin biopsy showed a leucocytoclastic vasculitis. Leucocyte count was $23 \times 10^9/l$ with 80% lymphocytes. A bone marrow sample contained 30% small mature lymphocytes.

Hepatic tests indicated a mild cholestasis without cytolysis. A monoclonal immunoglobulin was detected on serum electrophoresis. By immunofixation, it corresponded to IgGλ molecules; in addition there were small amounts of homogeneous IgMλ and IgGκ molecules. A number of autoantibodies were detected in this patient's serum: rheumatoid factors (480 U; normal <30), positive Coombs' test with anti-γ and comple-

ment reagents, polyclonal antinuclear antibodies up to a 10^{-3} dilution by immunofluorescence, antibodies to ssDNA by ELISA, a lupus-like circulating anticoagulant antibody and high titre anticardiolipin antibodies (320 U/ml; normal <20). The latter antibodies were of the IgM or IgG isotypes, and exhibited only λ light chains. Study of the complement system revealed a heterozygous deficit in C4B.

Establishment of heterohybrids

T cell-depleted blood cells were fused with the X63-Ag8.653 murine myeloma in the presence of 45% polyethylene glycol (mol. wt 4000) and 5% dimethylsulphoxide (DMSO). The supernatants of the resulting hybrids were screened for IgM and IgG secretion by ELISA; positive wells were cloned twice by limiting dilution.

Anticardiolipin and anti-DNA ELISA

Anticardiolipin antibodies were measured in an ELISA adapted from Koike *et al.* [6]. Briefly, microtitration plates were coated with 2·5 μg cardiolipin (Sigma, St Louis, MO) in 50 μl ethanol followed by evaporation for 2 h at 37°C. Plates were then saturated overnight at 4°C with PBS containing 2% bovine serum albumin (BSA). After five washes, 50 μl of serum of hybrid supernatant were added to each well (in triplicate) for 1 h at room temperature. After another five washes in PBS-BSA 0·05% Tween 20 (Merck, Darmstadt, Germany), antibodies were revealed by peroxidase-conjugated anti-μ, anti-α, anti-γ (Institut Pasteur Production, Paris, France), anti-κ and anti-λ (Tago Inc, Burlingame, CA) goat antibodies. Every plate included positive and negative control sera, and uncoated plates were used as control of non-specific binding to the plastic. The dependence of β_2 glycoprotein I (β2GPI) for binding to cardiolipin was assayed as described [7].

ELISA for detection of antibodies to ssDNA and dsDNA was performed as described [8].

Extraction of DNA and RNA

Total DNA and RNA were simultaneously extracted from 2×10^7 cells using the guanidinium isothiocyanate method [9].

Southern blot analysis

DNA (10 μg) was digested with EcoRI and BamHI restriction enzymes (Boehringer-Mannheim, Meylan, France), separated on 0·8% agarose gels and transferred to nylon membranes (Hybond N; Amersham, Aylesbury, UK) by the method of Southern [10]. Hybridization with hexamer-extended J_H and C_λ probes was accomplished and the filters were washed in 0·1X SSC, 0·1% SDS at 65°C with overnight exposure on Kodak XAR films using amplifying screens as reported before [11].

Construction of cDNA library

Enrichment for poly(A)+mRNA was obtained by processing total RNA sample through two successive oligo (dT) cellulose columns. One microgram of this purified material was used to synthesize cDNA using a cDNA synthesis kit (Pharmacia, Uppsala, Sweden). The primers consisted of a 17mer oligonucleotide specific for the 5′ end of Cμ (5′ACGCTCGTATCCGACGG3′), and a consensus 17mer specific for the 5′ end of the four functional Cλ: λ3, λ2, λ7, λ1 (5′GTGGCCTTGTTGGCTTG3′). Fragments of double-stranded cDNA were ligated into a dephosphorylated EcoRI digested M13-mp18. The ligation mixture was used to transform JM105 *Escherichia coli* competent cells, and the library was plated.

Cloning and sequencing

The colonies were screened with two 20mer oligonucleotides complementary to the Cμ, located 5′ to the primer and specific for each strand of the cDNA (5′CATCCGCCCCAACCCTTTTC3′ and 5′TTCTCACAGGAGACGAGGGG3′) and with four 20mer oligonucleotides complementary to the various Cλ in a region 5′ to the primer (5′CTCCTCAGAGGAGGGTGGGA3′, 5′CTCCTCAGAGGAGGGCGGGA3′, 5′AAGGCTGCCCCCTCGGTCAC3′ and 5′AAGGCCAACCCCACTGTCAC3′). Oligonucleotides were end-labelled with ^{32}P-gamma-ATP and polynucleotide kinase. Oligonucleotides were purchased from Genset (Paris, France).

Prehybridization and hybridization were performed at 56°C in 3 M tetramethylammonium chloride, 0·05 M $NaPO_4$ pH 6·8, 5X Denhardt, 0·6% SDS and 100 μg/ml salmon sperm DNA. Subsequently, filters were washed twice at 20°C for 30 min in 3 M tetramethylammonium chloride, 0·2% SDS, 50 mM Tris HCl pH 8, followed by 1 h wash with the same mixture at 50°C, and autoradiographed.

After subcloning, ssDNA was prepared from positive colonies and sequenced by the dideoxy chain termination procedure [12] using ^{35}S-alpha-thio ATP and T7 DNA polymerase.

Subsequences were compared with Genbank (release 76) and EMBL (release 34) sequence databases using the CITI 2 facilities.

RESULTS

Characterization of the antibody activity of monoclonal IgM produced by CLL cells

B cells from patient Ren were fused with the murine myeloma X63-Ag8.653 and the resulting IgM-secreting heterohybrids were cloned twice. By direct cytoplasmic immunofluorescence, cells from these hybrids produced only monotypic IgMλ molecules. Study of immunoglobulin gene configuration of four different hybrids showed identical gene rearrangements for both heavy and light chains. The size of the rearranged bands was identical to that observed on digests of peripheral blood cell DNA (Fig. 1). The latter finding indicated that the heterohybrids studied derived from fusion with the leukaemic B CLL cells. Screening of the supernatants for anticardiolipin antibodies was performed by ELISA. All supernatants contained high titre IgMλ anticardiolipin antibodies. The binding of these antibodies was dependent upon the β2GPI cofactor. IgM from supernatants also reacted with ssDNA, but not dsDNA. There was no binding to a panel of autoantigens which are frequent targets of polyspecific antibodies (human IgG, thyroglobulin, actin, myosin, cytoskeleton intermediate filaments) (data not shown).

Sequence analysis of the anticardiolipin IgM heavy chain

The heavy chain belonged to the V_H4 gene family. The most homologous V_H4 germline gene was the V71-2 gene [13] with 96·3% identity (Fig. 2). However, the 11 nucleotide changes (six in doublets) correspond to variations observed in other published V_H4 gene sequences [14,15], suggesting gene polymorphism rather than somatic mutations. The CDR3 region was 45

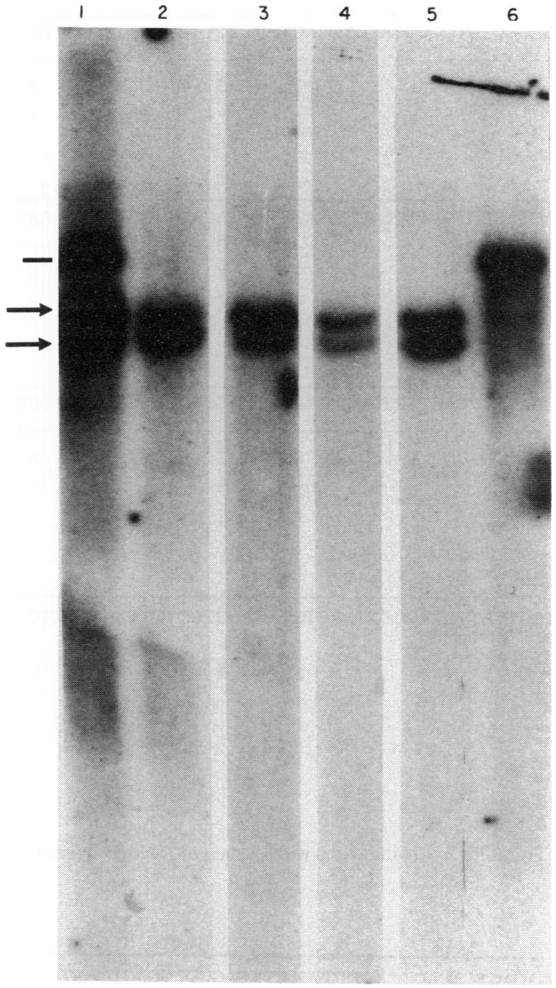

Fig. 1. Study by Southern blot of peripheral blood lymphocytes (PBL) and heterohybrids: the leukaemic cells from PBL (lane 1) and from hybrids (lanes 2–5) exhibit an identical rearrangement, as evidenced by hybridization with a J_H probe on EcoRI digests. Arrows indicate rearranged bands and (−) the germline band (lane 6: DNA from placenta).

bp long and was 100% homologous to the $D_{XP}4$ germline gene from bp 9 to 31 [16]. The 5′ and 3′ nucleotides probably corresponded to N nucleotide addition. The J_H segment was coded by the J_H3 segment, exhibiting a single difference with the germline gene at position 10, which is probably related to a polymorphism.

Sequence analysis of the anticardiolipin IgM light chain

The lambda light chain belonged to the new $V_\lambda 8$ variability subgroup and exhibited 99·7% homology with the single known germline $V_\lambda 8$ gene [15] (Fig. 3). There was one replacement mutation in the CDR1. The V_λ region was joined to a $J_\lambda 3$ segment and to the $C_\lambda 3$ gene.

DISCUSSION

This patient presented with CLL and a complex autoimmune syndrome featuring cutaneous vasculitis, fever, arthralgias, phlebitis, haemolytic anaemia and cholestatic hepatitis. A number of autoantibodies were present in his serum, i.e. rheumatoid factors, antinuclear antibodies, antibodies to erythrocytes, anticardiolipin antibodies, and lupus-like anticoagulant antibodies.

Serum antibodies to cardiolipin were restricted to IgGλ and IgMλ molecules. Since surface immunoglobulin of CLL cells were of IgMλ isotype, we wondered whether in this particular patient the leukaemic cells could produce this autoantibody, which probably had a pathogenic role in the patient's disease. It had long been recognized that there is usually an arrest in the maturation of CLL cells to plasma cells [18]; however, in all patients there is a small amount of serum monoclonal IgM (and IgD) idiotypically related to the surface immunoglobulin synthesized by CLL cells [19]. We found that heterohybrids produced with patient's leukaemic cells produced IgMλ molecules reactive with cardiolipin and ssDNA. The former activity was dependent upon the presence of β2GPI cofactor, a finding indicative of pathogenic anticardiolipin antibodies [7,20]. It is tempting to speculate that the restricted IgGλ antibodies to cardiolipin found in serum were clonally related to the leukaemic clone, as occurs in some lymphoplasmocytic diseases [21]; we have at present, however, no data to verify this hypothesis, since there were no IgG-secreting hybrids.

Most, if not all, so far identified antibody activities supported by CLL immunoglobulin belong to the category of natural, polyspecific antibodies [1,2]. This was not the case in our patient; the reactivity with both ssDNA and cardiolipin is explained by similar epitopes, and the hybridoma-derived IgM did not react with the usual targets of polyreactive antibodies. Pathogenic autoantibodies observed in CLL patients are mostly responsible for haemolytic anaemia or thrombocytopenia. As discussed above, these antibodies are not synthesized by CLL cells. In the present patient, the thromboembolic complication and vasculitis could well be related to the clonal anticardiolipin antibodies, as occurs in systemic lupus erythematosus (SLE) [7]. Of note, our patient has no evidence of systemic autoimmune disease such as lupus. The existence of a heterozygous C4B deficiency may be coincidental in view of its notable incidence in normal individuals. We cannot, however, rule out that it had a role in the development of this unusual immunologic disorder.

CLL B lymphocytes are generally believed to use a restricted repertoire of V_H and V_L immunoglobulin genes; the small V_H families V_H4, V_H5, V_H6 are overrepresented in this repertoire [22,23]. The presence of somatic mutations in V_H and V_L genes of CLL B cells is controversial. It is generally believed that these genes retain a germline configuration [24,25]; however, Cai *et al.* [26] demonstrated the presence of somatic mutations in V_H5 251 genes with a high R/S ratio in complementarity-determining regions (CDRs), indicative of an antigen-driven process. Nevertheless, opposite results were recently reported, the same V_H5 251 gene being in germline configuration in CLL [27]. On the other hand, polyclonal antibodies such as anti-DNA, -IgG, -lamin [17,28,29], in autoimmune diseases used diverse and often mutated V_H and V_L genes, suggesting an antigen-driven process.

In this context, it was of interest to identify and sequence the V_H and V_L genes encoding IgM MoAbs to cardiolipin. The heavy chain of Ren CLL B lymphocytes is encoded by a V_H4 gene. Although the corresponding germline gene has not been sequenced in our patient, V_H Ren probably derives from an allotype variant of the V71-2 gene. This gene, or one of its

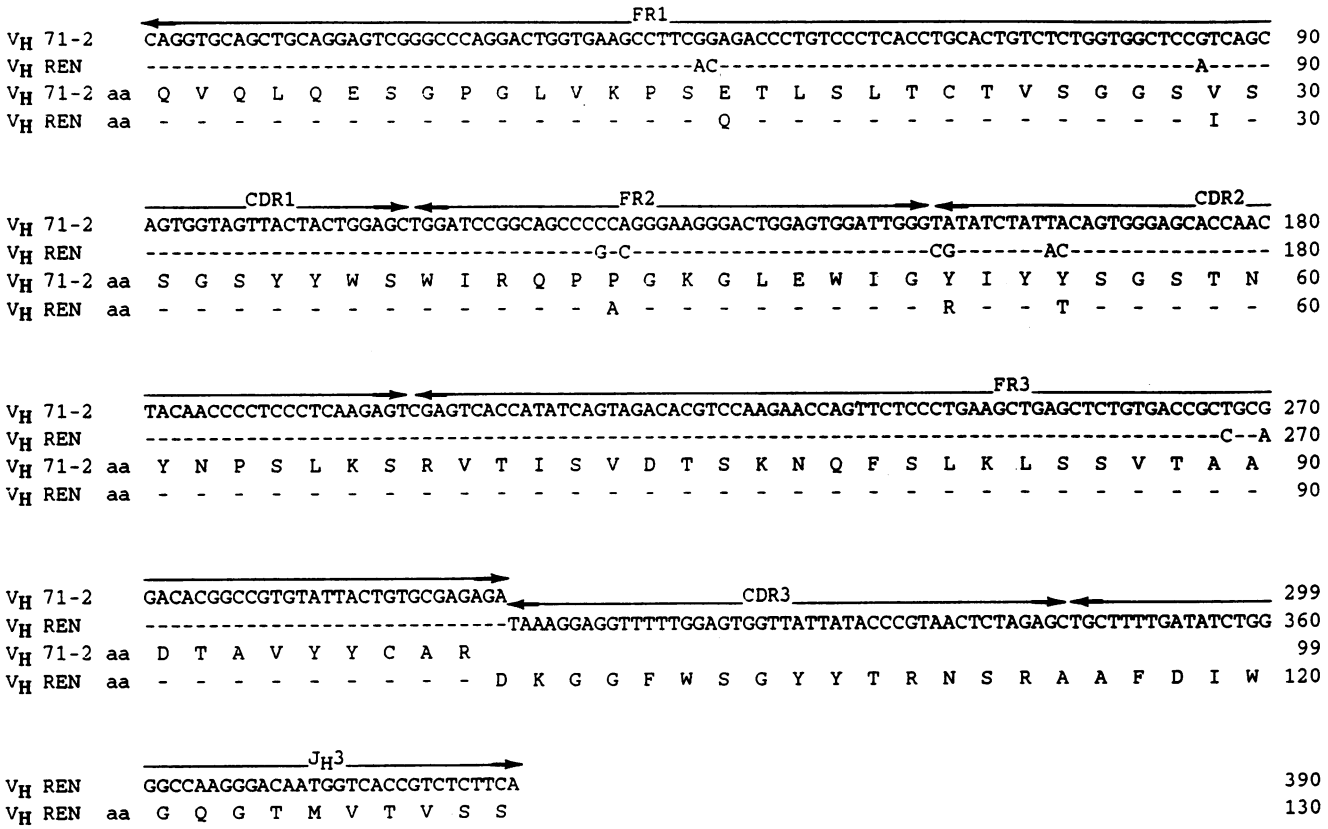

Fig. 2. Sequence of V_H Ren aligned with that of the V_H4 71-2 germinal gene. Homologies are indicated with dashes. aa, Deduced amino acid sequence.

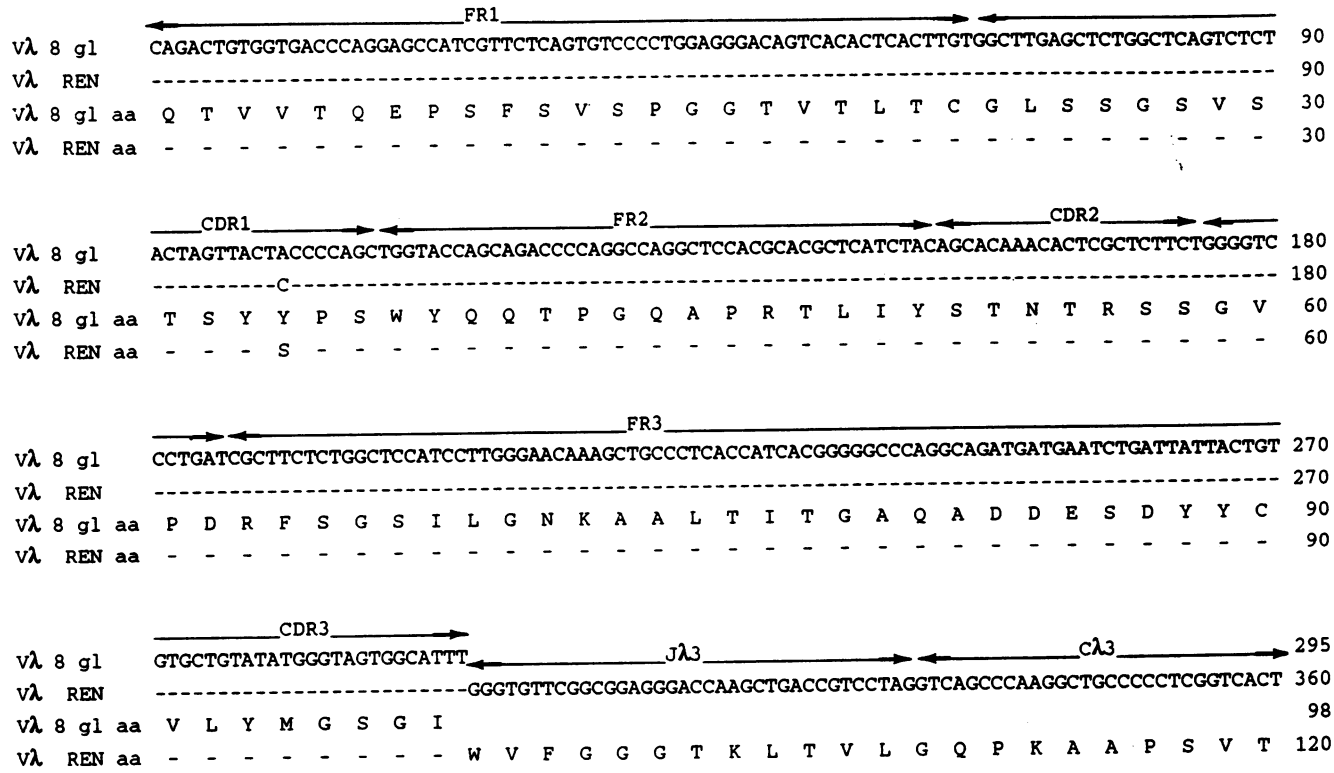

Fig. 3. Sequence of V_L Ren aligned with the single $V_\lambda8$ germinal gene. Homologies are indicated with dashes. aa, Deduced amino acid sequence.

variants, has already been used to encode the heavy chain of immunoglobulin from another B CLL [30], and of other autoantibodies such as rheumatoid factor, anti-dsDNA antibody, anti-thyroglobulin antibody and a natural anti-DNA/cardiolipin antibody [31]. These latter data point to the importance of the CDR3 sequence which is crucial for antigen binding, and which may modulate the specificity of an antibody encoded by a given V_H (and V_L) gene [32].

The V_H4 family has been extensively studied at the germline level in different individuals and in various countries [13–15,33–35]. Several genes of this family such as $V_H4.21$ exhibit little or no polymorphism [33–35]; accordingly the V71-2 gene is 100% identical to the H1 germline gene sequenced in an unrelated individual [34,35]. However, two recent reports describe allotypic variants of V_H4 genes, notably of V_H 71-2 [14,15]. Close examination of published sequences revealed that all nucleotide changes (including six in doublets) noted between V_H Ren and V71-2 are present in some allotypic variant genes related to V71-2, V2-1, V58 or V4-35. Thus, these nucleotide changes are unlikely to be related to somatic mutations, but rather reflect the use of a new germline gene or of an allotypic variant of the V71-2 gene. This would be a further example of the diversity of the V_H4 family, which may arise in large part by the acquisition of blocks of nucleotides [15].

The light chain of the IgM Ren belongs to the $V_\lambda8$ family. It exhibits 99·7% homology with the single germline gene of this new family, with a single replacement mutation in the CDR1. Interestingly, the only known antibody using this $V_\lambda8$ gene had an anti-dsDNA activity in a patient with SLE [17]. It would be of interest to investigate whether the $V_\lambda8$ gene may be used by other anticardiolipin/β2GPI antibodies.

At present there are few data on the structure of anticardiolipin antibodies. Six anticardiolipin antibodies sequenced to date are polyreactive and belong most probably to natural antibodies; they use various V_H genes exhibiting few somatic mutations: V_H6 (L16/ML1 and A10/A431) [36], V_H1 51p1 (Kim 13.1) [37], V_H26 (member of the V_H3 family) (18/2) [38], V_H3 1.9 III (Kim 4.6) [5], V_H4 V71-2 (C6B2) [39]. Two V_L genes were identified and derived from unmutated $V_\kappa3$ [37] and $V_\lambda1$ [5] germline genes. Interestingly, the V_H1 51p1 and the $V_\kappa2$ genes used by another anticardiolipin antibody derived from a patient with active SLE displayed antigen-selected somatic mutations [40]. No such evidence was found in our patient with B CLL secreting a monoclonal anticardiolipin/β2GPI autoantibody who featured thrombosis and vasculitis, which are sometimes associated with pathogenic anticardiolipin/β2GPI antibodies. The existence of a peculiar clinical entity associated with anticardiolipin/β2GPI autoantibodies, characterized by lupus-like and thromboembolic manifestations, legitimizes genetic studies of this variety of autoantibody.

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