

Pathogenic antiphospholipid antibody: an antigen-selected needle in a haystack

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Antiphospholipid antibodies represent a heterogeneous group of autoantibodies directed against anionic phospholipids (PLs) usually linked to protein cofactors. Their presence during the antiphospholipid syndrome is associated with risks of thrombosis and fetal losses. Among 5 randomly selected monoclonal antiphospholipid antibodies, all originating from a single patient suffering from this autoimmune dis-

ease, only 1 induced fetal losses when passively injected into pregnant mice. Its antiphospholipid activity was dependent on annexin A5, and its variable regions contained mainly 3 replacement mutations. To clarify the role of these mutations in the pathogenicity of the antibody, they were in vitro reverted to the germ line configuration. The resulting "germ line" antibody reacted with multiple self-antigens and only partially

lost its reactivity against PLs, but it was no more dependent on annexin A5 and, more importantly, was no more pathogenic. This study illustrates that the in vivo antigen-driven maturation process of natural autoreactive B cells can be responsible for pathogenicity. (Blood. 2004;104:1711-1715)

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Introduction

Antiphospholipid antibodies (aPLs) constitute a heterogeneous set of autoantibodies directed against a variety of anionic phospholipids such as cardiolipin (CL) or phosphatidylserine. This heterogeneity is obvious at different levels: (1) the autoantibody fine specificity is widened by the cofactor dependency, which indicates the frequent requirement of a variety of phospholipid (PL) binding serum proteins named cofactors (such as β 2-glycoprotein I, prothrombin, protein C, protein S, or annexin A5) for the antibody reactivity¹⁻⁶; (2) the molecular structure of the variable regions of aPLs is extremely diverse⁷⁻⁹; and (3) APLs are secreted not only during autoimmune pathologies such as the antiphospholipid syndrome (APS), which is characterized by recurrent life-threatening arterial and venous thrombosis as well as recurrent fetal losses, but also under nonautoimmune conditions, such as infectious diseases, or even in healthy young adults without causing any thrombotic complication.

The links between normal and pathologic aPLs are not well understood. In particular, the appearance of these antibodies in physiological conditions leads to 3 main questions: What are the precise stimuli that induce the aPL production? What is the role, if any, of these autoantibodies? How is their production controlled? Different data suggest that aPLs are able to recognize phosphatidylserine (PS) when exposed at the outer part of the cell membrane during the early phase of apoptosis.¹⁰ Indeed, it seems that mouse aPLs can be positively selected but also that aPL-producing B cells stay under the control of tolerance mechanisms such as receptor editing.^{11,12} In human beings, aPL

B cells are present in the normal peripheral repertoire, with a discrete pool of memory B cells able to produce somatically mutated forms of these antibodies.¹³ According to the current hypothesis, normal aPLs, owing to their ability to bind anionic PLs, participate in the clearance of apoptotic cells and apoptotic debris.¹⁴ On the other hand, during the autoimmune disease APS, some of these antibodies are known to be pathogenic because they can reproduce fetal losses or thrombosis when passively transferred in animal models.¹⁵⁻¹⁸ Are these pathogenic aPLs derived from physiologic ones?

Considering such heterogeneity of aPLs, it is important to define the precise characteristics of pathogenic aPLs. To approach this question, we randomly selected 5 different monoclonal aPLs (maPLs) originating from a single patient suffering from an APS⁷ and passively transferred each one of these maPLs into pregnant mice. Only one of these maPLs was pathogenic by inducing fetal resorptions. Its antibody variable regions mainly contained 3 mutations, which were shown to be directly linked to the acquisition of pathogenicity.

Patient, materials, and methods

Patient

The patient was a 27-year-old woman suffering from a primary APS. She presented with 6 recurrent spontaneous miscarriages and 2 episodes of deep vein thrombosis of the legs within the last 2 years. The patient's serum consistently contained marked elevated levels of aPL immunoglobulin G (IgG) (enzyme-linked immunosorbent assay [ELISA], 84 units GPL) and a

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weak and transient lupus anticoagulant. After obtaining informed consent from the patient, 30 mL blood was drawn and was used for single IgG aPL cell sorting as described.⁷

maPLs

The maPLs were obtained as described.⁷ Briefly, single aPL B cells from the patient's peripheral blood were sorted by flow cytometry using cardiolipin-labeled vesicles. Messenger RNAs of these single B cells were subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) to amplify the V region genes of the H and L chains. The amplification products were cloned into recombinant baculovirus to produce monoclonal human Ig1K in insect cell (sf9) cultures. Monoclonal antibodies CIC3, CIC7, CIC11, CIC15, CIC19, and GL were then purified from the culture supernatants on columns of protein A–Sepharose according to the manufacturer's instructions (Sigma, St Louis, MO).

ELISAs for aPL reactivity, cofactor dependency, and autoantibody profiles

aPL reactivity of CIC7 and CIC15 was tested as previously described, as was cofactor dependency.⁷ The autoantibody profile was tested by using purified human thyroglobulin, human myoglobin, and purified ssDNA as previously described.¹⁹ The binding of CIC15 to annexin A5 or to annexin A5-CL complexes was tested by ELISA: Purified delipidized recombinant annexin A5 (gift from J. M. Freyssinet, Strasbourg, France) or complexes of previously incubated annexin A5 and CL—5 $\mu\text{g}/\text{mL}$ annexin A5 and 50 $\mu\text{g}/\text{mL}$ CL—in Tris (tris(hydroxymethyl)aminomethane)–buffered saline (TBS) containing CaCl_2 were allowed to bind to plastic plates (Maxisorp, Nunc, Roskilde, Denmark). Plates were saturated with delipidized bovine serum albumin (BSA) (Roche Diagnostic, Mannheim, Germany), and then various concentrations of CIC15 or normal human IgG in TBS containing 2 mM CaCl_2 were added for 2 hours at 37°C before peroxidase-labeled anti-human IgG (1:15 000), (Jackson ImmunoResearch Labs, West Grove, PA) was added.

Production of the germ line counterpart of CIC15

The PCR products of the CIC15 V regions originating from a single IgG aPL B cell were gel purified and sequenced with the primers used in the second round of PCR amplification and the Big Dye Terminator cycle DNA sequencing kit (Applied Biosystems, Foster City, CA) on an automated ABI 310 sequencer (Applied Biosystems). The V region genes of CIC15 were analyzed using the international ImMunoGeneTics (IMGT) information system, which provides integrated knowledge resources specializing in the immunoglobulin sequence comparisons (<http://imgt.cines.fr/textes/IMGTcomparesequence.html>).²⁰

The 3 replacement mutations present in the first hypervariable region of CIC VK were reversed to the germ line configuration by site-directed mutagenesis using 3 sets of PCR. PCR1 used 2 primers, GACATTGAGCTCACCAGTCTCCA (α , sense) and GCCAACCAGTACTAATACTCTGACTGG (antisense), and PCR2 used 2 other primers, CGAGTCAGAGTATTAGTAGCTGGTTGGC (sense) and GGGACCAAGCTCGAGATCAAACGG (β , antisense). The products of PCR1 and PCR2, which introduced the desired mutations, were mixed and amplified (PCR3) using α and β oligonucleotides containing *SacI* and *XhoI* cloning sites (underlined). The products of the PCRs were sequenced to verify the changes and cloned again into the baculovirus with CIC heavy chain V region. The germ line counterpart of CIC15 was then produced by infected sf9 cells and purified from the supernatant.

Model of Ig transfer into pregnant mice

aPL monoclonal antibodies (2 \times 100 μg) or control normal human IgG (Novartis, Basel, Switzerland) were intravenously injected into the tail vein of female mice (BALB/c, 2 to 4 months) at day 1 and day 2 postcoitum. The serum level of human IgG present in these mice was tested 1 hour after the second injection to check for the quality of the injections. The pregnant animals were then killed at day 10 postcoitum for macroscopic examination

of the embryos. Fetal resorptions were defined as 50% reductions of the normal fetal volume.

Molecular models

Tridimensional models of CIC15 and its germ line counterpart (GL) were generated using SWISS-MODEL, a protein structure homology-modeling server accessible via the program DeepView/swiss Pdb-viewer (<http://www.expasy.org/spdbv>).^{21,22} Known 3-dimensional structures (obtained by x-ray crystallography or nuclear magnetic resonance spectroscopy) were used as templates: 1fgvL.pdb, 1bv1B.pdb, and 1f3rB.pdb for CIC15 and 1hezA.pdb for GL; the sequence similarities of the templates to the target sequences are high, especially in the region of the light chain complementarity-determining region-1 (CDR1) loop where the 3 mutations are localized (73% to 82% homology).

Results

The purified IgG fraction from the serum of the patient contained polyclonal aPLs with various cofactor dependencies⁷ and pathogenic antibodies as judged by its capacity to induce fetal losses and fetal resorptions in a model of passive transfer into pregnant mice (data not shown). We took advantage of our previous analysis of the CIC aPL activity at the clonal level to track pathogenic autoantibodies among the polyclonal aPLs present in the serum.⁷ The variable regions of the heavy and light chains of the CIC aPL single-sorted B cells were cloned into a recombinant baculovirus to produce human monoclonal IgG1Ks in insect cell cultures. We randomly selected the first 5 maPLs that were produced in large enough quantities to test their pathogenicity after passive transfer in the murine model of pregnant mice. The reactivity and the cofactor dependency of 3 monoclonal antibodies (CIC3, CIC11, and CIC19) have already been described.⁷ Briefly, these 3 monoclonals react with both PS and CL; CIC3 is mainly dependent on β 2-glycoprotein I, CIC11 is mainly dependent on protein C, and CIC19 is dependent on both prothrombin and protein S. The characteristics of CIC7 and CIC15 are described in Figure 1.

Only one maPL is able to induce fetal losses and fetal resorptions in mice

Female mice were intravenously injected at day 1 and day 2 postcoitum with 100 μg maPLs (groups of 5 to 10 pregnant mice per maPL or control human IgG). At day 2, the serum levels of the human IgGs reached approximately 18 $\mu\text{g}/\text{mL}$. The animals were killed and examined at day 10. By looking at the rosaries of embryos, we observed that CIC15 alone was able to induce fetal resorptions in a significant manner ($P = .02$, CIC15 versus normal human IgG) (Figure 2). Macroscopic examinations of the mice injected with CIC15 did not reveal any vascular thrombosis (data not shown).

Characteristics of the maPL CIC15

We precisely analyzed the characteristics of the pathogenic maPLs and showed that, in presence of fetal calf serum (FCS), CIC15 reacted only with cardiolipin but not with another anionic PL (PS) or with a neutral PL (phosphatidylcholine [PC]) (Figure 1A). This cardiolipin reactivity is dependent on the presence of annexin A5 as protein cofactor but not on β 2-glycoprotein I, protein C, protein S, or prothrombin (Figure 1B). CIC15 was able to bind delipidized recombinant annexin A5, but its reactivity was enhanced by the presence of CL, showing that CIC15 mainly recognized the complexes formed between CL and annexin A5 (Figure 1C).

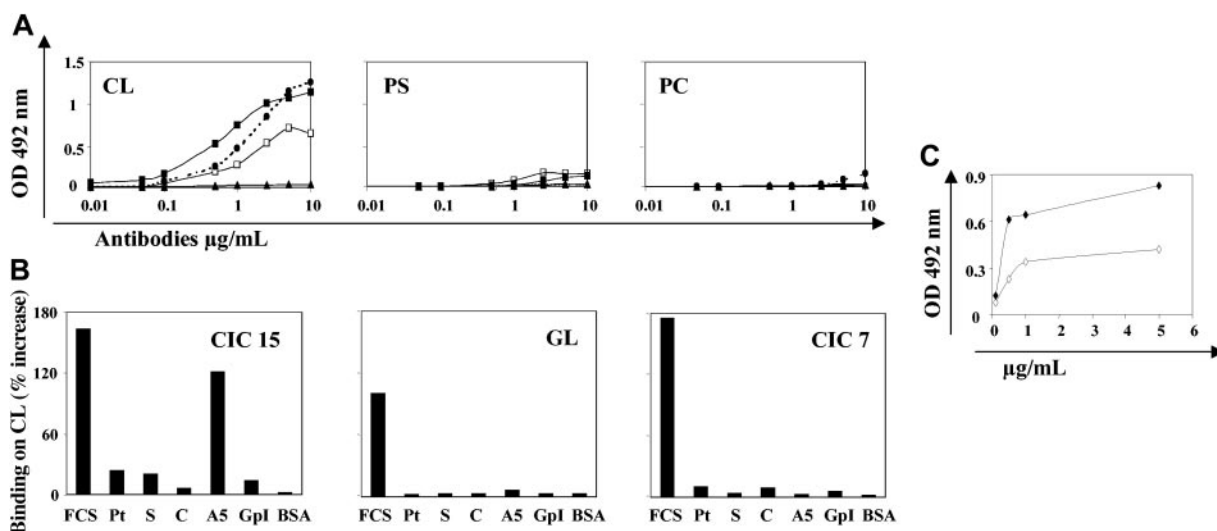


Figure 1. Reactivities and cofactor dependencies of CIC7, CIC15, and GL. See "Results." (A) Reactivity with CL, PS, and PC during ELISA assays in presence of FCS, CIC15 (■), CIC7 (●), GL (□), and normal human IgG (▲). (B) Cofactor dependency of CIC15 and GL anti-CL reactivity: Results are expressed as percentages of the increase of binding on CL in presence of FCS, prothrombin (Pt), protein S (S), protein C (C), annexin A5 (A5), β 2-glycoprotein I (Gpl), and BSA compared with the binding to CL without the cofactors. (C) Binding of CIC15 on delipidized recombinant annexin A5 (◇) or complexes of CL-annexin A5 (◆).

Finally, the molecular analysis of CIC15 revealed that this antibody was made of almost unmutated heavy chain variable regions (VH, 2 replacement mutations in the first framework, compared with the closest germ line, IGVH4-61*02) but mutated VK regions (closest germ line, IGKV1-5*03) (Figure 3).

We confirmed that the putative VK germ line was the actual patient's germ line by sequencing it from the patient's polynuclear leukocyte DNA (not shown). The VK mutations are grouped together in the CDR1 of the VK domain, replacing 3 serine residues by asparagine residues, which are commonly described among aPL

antibodies so far analyzed.⁹ These replacement mutations in a CDR of an antibody V region are usually considered to be a hallmark of an antigen selection process that affected the producing B cell.

For comparison, it is interesting that the previous molecular analysis of the nonpathogenic CIC11 VH region also revealed mutations located in a way that suggested an antigen-driven maturation process (replacement/silent mutations in the CDRs versus frameworks, $P < .05$; see Lieby et al⁷). On the contrary, the same analysis of VH and VK regions of CIC3 and CIC19 did not suggest such an antigen-driven process.

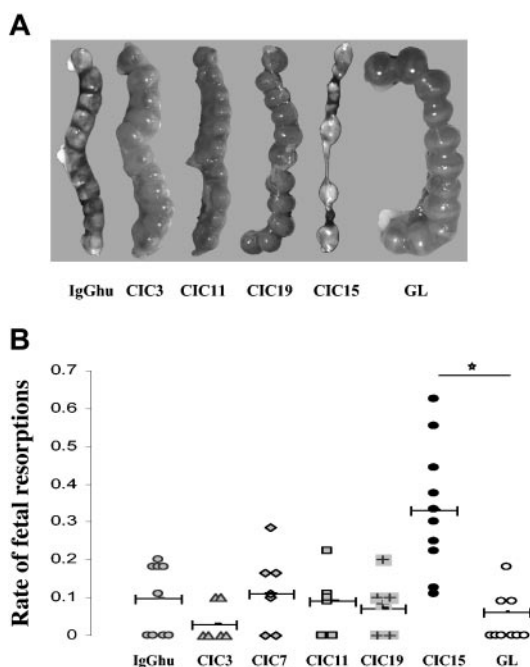


Figure 2. Effects of CIC maPLs on mouse pregnancy development. (A) Examples of macroscopic examinations of mouse embryos at day 10 after passive administration of maPLs or normal human IgG. (B) Rates of fetal resorptions after passive transfer of normal human IgG (n = 6), CIC3 (n = 7), CIC7 (n = 7), CIC11 (n = 6), CIC19 (n = 6), CIC15 (n = 10), and GL (n = 9). Horizontal brackets indicate mean values; horizontal bar with the star, statistical difference ($P < .001$).

The mutations are responsible for the pathogenicity

The antibody fine specificity is mainly linked to the structure of its H and L CDRs, with a major impact of the H CDR3.¹⁹ Thus, to precisely define the role, if any, of these 3 somatic mutations in the pathogenicity, we reversed the CIC15 VK sequence to the germ line configuration to produce the "germ line" counterpart (GL) of CIC15. The GL antibody, which differs from CIC15 only by the 3 residues in the VK CDR1, partly lost its activity against cardiolipin but still reacted with this antigen in presence of FCS (Figure 1A). However, GL was no more dependent on annexin A5 (Figure 1B) and, more importantly, was no more pathogenic when passively administered to pregnant mice with serum levels equivalent to those obtained with CIC15 (percentage of fetal resorptions, 6%; GL versus CIC15, $P < .0001$) (Figure 2). Thus, the naturally occurring in vivo replacement of the 3 serine residues by 3 asparagines in the VK CDR1 maintained the general structure of the CDR1 loop and its flexibility (see the 3-dimensional modelizations in Figure 4) but also created the chemical conditions that allowed CIC15 to react with the PL-annexin A5 complexes. The replacement of serine by the more bulky asparagine in CIC15 introduces subtle changes in the 3-dimensional structure of the loop: In particular, 3 more nitrogen atoms are available to form hydrogen bonds with ligands, and the orientation of the amino acids flanking the mutations is modified. These changes were directly responsible for the observed CIC15 pathogenicity.

Finally, it is also interesting that the GL antibody has an autoreactivity profile that is similar to the known multireactivity of

IGHV4-61*02	CAGGTGCAGCTGCAGGAGTCGGGCCAGGACTGGTGAAGCCCTCACAGACCCCTGTCCTCACCTGCATGTCTCT	75	
CIC15	-----G-----G-----		
	CDR1		
IGHV4-61*02	GGTGGCTCCATCAGCAGTGGTAGTTACTACTGGAGCTGGATCCGGCAGCCCGCGGGAAGGGAAGTGGAGTGGATT	151	
CIC15	-----		
	CDR2		
IGHV4-61*02	GGGCGTATCTATACCAGTGGGAGCACCACTACAACCCCTCCCTCAAGAGTCSAGTCACCATATCAGTAGACAC	225	
CIC15	-----		
IGHV4-61*02	GTCGAAGAACCACTTCTCCCTGAAGCTGAGCTCTGTGACCCGCGCAGACACGGCCGCTGTATTACTGTGCGAGA	298	
CIC15	-----		
	CDR3		
CIC15	GATAAAGGAGGTTTTTGGAGTGGTTATTATACCCGTAACCTCTAGA	343	
JH4*03	GCTACTTTGACTACTGGGCAAGGGACCCCTGGTCAACCTCTCCTCA	390	
IGKV1-5*03	GACATCCAGATGACCCAGTCTCCTCCACCCTGCTGCACTCTGTAGGAGACAGAGTCAACCATCACTTGCCGGGCCAGT	78	
CIC 15	-----		
	CDR1	CDR2	
IGKV1-5*03	CAGAGTATTACTAGCTCT	TTGGCTGGTATCAGCAGAAACCAGGAAAGCCCTTAAGCTCCTGATCTATAAGCGCTCT	156
CIC15	---A---A---A---	-----	
IGKV1-5*03aa	---Ser---SerSer---	-----	
CIC15aa	---Asn---AsnAsn---	-----	
IGKV1-5*03	AGTTTAGAAAGTGGGTCCTCAAGGTTTCAGCGCCAGTGGATCTGGGACAGAATTCACCTCTCACCATCAGCAGCCTGCAG	237	
CIC15	-----C-----		
IGKV1-5*03	CCTGATGATTTTGCACCTTATTACTGC	264	
CIC15	-----t-----		
	CDR3	IGKJ2*02	
CIC15	CAACAGTATAATAGTTATTTCAG	GACGTTCCGGCCAGGGACCAAGCTCGAGATCAAACG	322

natural autoantibodies, binding to human thyroglobulin, human myoglobin, and ssDNA. On the contrary, CIC15 multireactivity is much less obvious (Figure 5).

Discussion

Animal models of passively transferred polyclonal or monoclonal Ig with aPL reactivity have previously been reported¹⁵⁻¹⁸ and clearly demonstrated the potential pathogenicity of aPLs. Salmon and coworkers, using polyclonal aPLs, established that the activation of the complement C3 was of considerable importance in the effector phase of the pathogenicity using this model.²³ Here, we show that the pathogenic antibody CIC15, which is able to reproduce the patient’s obstetrical pathology, is “hidden” among nonpathogenic aPLs in the patient’s serum. Indeed, the classical detection of aPLs takes advantage of the patient’s serum and FCS-containing cofactors to reveal the general aPL reactivity but is unable to precisely detect the “few” pathogenic antibodies. It is interesting that, using a classical cofactor-dependency test with the patient’s total serum, we were almost unable to detect annexin A5-dependent antibodies.⁷ This is probably the result of the low sensitivity of this assay. Such a detection of clinically relevant antibodies in women with recurrent fetal losses is an important goal for physicians, but the target could be difficult to reach without designing new tests, in particular if the relative amount of the pathogenic antibody is low compared with the others.

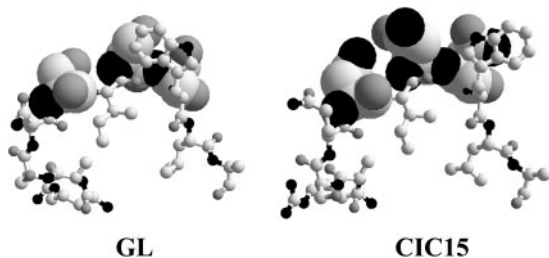


Figure 4. Tridimensional models of CIC15 and its germ line counterpart (GL). Only the amino acids of the VK CDR1 loop containing the 3 mutations (serine → asparagine) are shown here. Balls and sticks represent the unmutated amino acids, whereas the “space-filling” representation (van der Waals surface) highlights the mutations. Atoms: black, N; dark gray, O; and light gray, C.

Figure 3. Molecular aspects of CIC15 and GL. Nucleotide sequences of CIC15 VH and CIC15 VK regions compared respectively with their closest VH and VK germ lines. CDRs are indicated; the box contains the VK CDR1 with the 3 replacement mutations distinguishing CIC15 from GL. European Molecular Biology Laboratory (EMBL) accession numbers of CIC15 VH and VK regions are AJ576037 and AJ576038, respectively.

CIC15 is probably not the only pathogenic aPL present in our patient, but it was shown to be dependent on annexin A5, and this peculiarity is probably linked to its pathogenicity. Among the first 14 maPLs originating from the same patient, only CIC15 was dependent on annexin A5 (data not shown). Different arguments highlight the role of annexin A5 in the placenta: This protein, which binds anionic PL (PS and CL) with a high affinity, is strongly expressed by placental trophoblasts and syncytiotrophoblasts under 2-dimensional crystalline arrays,²⁴ and the removal of annexin A5 from these cells creates the conditions for coagulation activation.²⁵⁻²⁷ Furthermore, injection of antiannexin A5 antibodies in pregnant mice also results in fetal losses.²⁷ Using these arguments and others, Rand suggested that the high-affinity binding of aPLs to PL-protein complexes sterically interferes with the ability of an ordered crystal structure of annexin A5 to form on the cell surface.²⁷ Under this hypothesis, aPLs could induce coagulation by disrupting the ordered structure of the annexin A5 shield, thereby

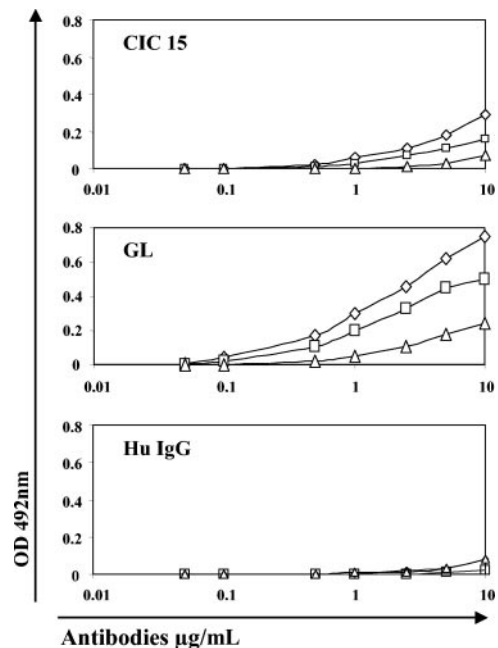


Figure 5. Autoantibody profiles of CIC15, GL, and normal human IgG. ◊ indicates human thyroglobulin; ◻, human myoglobin; and ◻, ssDNA.

increasing the availability of PL for coagulation reactions. In future experiments using CIC15, it will be interesting to test the precise placental localization of the antibody after passive administration into pregnant mice.

But the most important part of our present data concerns the conclusive demonstration that the affinity maturation process of a natural autoantibody can lead to pathogenicity in human disease. After introduction of a foreign antigen, the usual localization where affinity maturation of antibodies takes place is the germinal center of the secondary lymphoid organ. The random introduction of the mutations along the variable regions of the B-cell antigen receptor (BCR) leads naive B cells to a double process of positive and negative selections. B cells will receive a selective advantage for proliferation if the mutations increase the BCR affinity for the selecting antigen. On the other hand, they will die if the mutations have been neutral or even reduce the BCR reactivity. In normal conditions, this process, which could induce the appearance of autoreactive B cells, is controlled by peripheral tolerance mechanisms to avoid pathologic autoimmunity.²⁸⁻³⁰ Our results clearly show that this was not the case in our patient, because the germ line version of CIC15, initially present under a multi-self-reactive BCR form on a naive B cell emigrating from bone marrow, was able to

change its structure, escape peripheral tolerance mechanisms, and induce pathological consequences.

What could have been the driving forces that created such clinically important changes? Starting from the physiological presence of aPL B cells in human beings, repetition of normal stimuli (apoptosis) may induce the appearance of mutated forms of aPLs and the constitution of a memory B-cell pool.¹³ This B-cell pool is maintained in a state of immunologic ignorance, likely because of the still quite low affinity of these aPLs. Two circumstances could lead to the production of potentially pathogenic aPLs from this B-cell pool. In the first scenario, new stimuli could create the conditions that introduce additional mutations in the V regions of aPLs. For instance, systemic infections can both induce apoptosis of a variety of cells and stimulate, in particular, memory B cells through the constitutive expression of their Toll-like receptors.³⁰ Under this scenario, intrinsically normal peripheral tolerance mechanisms are just overloaded and lose the control of potentially pathogenic B-cell clones. The catastrophic APS, an uncommon variant of APS, could respond to this scenario knowing the role of infections in precipitating the disease. A second scenario puts the light on primary abnormalities of the peripheral tolerance mechanisms (genetic defect?) without the need for a burden of stimuli.

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