Physical properties as antigenic determinant: the example of antiphospholipid antibodies

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Abstract

Although the specificity of an antibody grows through process requiring somatic mutation, the Ag-binding polyspecificity of an antibody enlarges the Ag detection of the immune system. While the polyspecificity of antibody is its ability to bind at least two different Ags, apparent polyspecificity could also depends of characteristics of Ag considered. Whichever the type of epitopes, conformational or linear, they are commonly determined by the surface of the involved epitope residues. We demonstrate that the apparent polyspecificity of antiphospholipid antibody responsible for the antiphospholipid syndrome is due to an epitope characterized by the physical properties of amino acids whose polarity, hydropathy and steric volume but not by the sequence of residues. The physicals properties are, per se, the epitope for certain antibody. Although this novel concept provides insights on how the polyspecificity of an antibody can be linked up under the lead of a common motif of physical properties.

Key Words: Polyspecificity antibody, Antigenic specificity. Structural motif, Antiphospholipid syndrome, Antiphospholipid antibodies
Summary Table

“What is known on this topic”

- Antibody heterospecificity or polyreactive arises when an antibody reacts with a another and apparently unrelated antigens than with the one used to raise the antibody. Thus, an antibody could appear to have been obtained through an antigen with which it is unable to react.

- While the main pathogenic cause is described as the interaction of antiphospholipid antibodies (aPL) with the plasma protein β2-Glycoprotein 1 (β2GP1), the antigenic specificity of aPL is the subject of intense investigation.

“What does this paper add?”

- The physicals properties are, per se, the epitope for certain antibody.

- We confirm a new concept providing insights on how the different epitopes can be linked up under the lead of a common motif of physical properties highlighting additional ways to determine or predict antigenic determinant of certain antibodies.
Introduction

The antiphospholipid syndrome (APS) is an auto-immune disease associated with arterial or venous thrombosis and/or recurrent fetal loss. While the main pathogenic cause is described as the interaction of antiphospholipid antibodies (aPL) with the plasma protein β2-Glycoprotein 1 (β2GP1), the antigenic specificity of aPL is the subject of intense investigation. While of the five domains identified for β2GP1, strong evidence shows that the most relevant epitope (R39-R43) is a cryptic and conformation-dependent structure on the β2GP1-Domain I (β2GP1-DI). Nevertheless, although anti-β2GP1-DI antibodies seems to carry a relevant epitope, anti-β2GP1-DII-V antibodies have also been described. Adding to this controversy, members of the Toll-like receptor (TLR) family and the platelet receptor apolipoprotein E receptor 2' (apoER2'), a receptor belonging to the low-density lipoprotein receptor (LDL-R) family, as well as GPIIbα, were identified as putative candidates for aPL recognition (1).

While it has been often suggested that further studies are required to clarify the polyspecificity of aPL, it was recently shown that the structural motifs in the molecule appear to be more important for aPL binding than the specific amino acid sequences (2). This motif (ϕϕζζFxϕ) is primarily characterized by the polarity of the amino acids, in which ϕ represents non-polar residues (AVILMFWCPG) and ζ polar residues (YTSHKREDQN). In a less extent, the interactions are sensitive to the volume and the hydropathy of the amino acids. In the present study, we have generated a library of 44 peptides composed by different amino acids relative to the motif (ϕϕζζFxϕ) and tested their reactivity with aPL. We thus confirm a new concept providing insights on how the different epitopes can be linked up under the lead of a common motif of physical properties highlighting additional ways to determine or predict antigenic determinant of certain antibodies.
Results

To further confirm that aPL interacting motif is pleiotropic, we have generated 44 different peptides containing the motif φφφξξFxC and φφφξξFxφ as well as the φxFξξφφφ in which φ represents nonpolar residues (AVILMFWCPG), ξ polar residues (YTSHKREDQN), F represent phenylalanine and “x” is for any amino acid. We have then generated a protein array by coating these peptide on Streptavidin Coated High Capacity Plates 96 well plates. Using these protein arrays, we have tested and quantified the ability of the peptides to interact with aPL. As presented in Figure 1A, left panel, we can observed that all peptides containing the described-motifs are recognized by aPL although their binding abilities differ from the epitope R39-R43 (Figure 1A, asterisk). Among these recombinant peptides, 11 have the same percentage of binding than aPL-described epitope (R39-R43, Figure 1A, asterisk). “High binding” ability defined peptides without statistical difference with R39-43 epitope but possessing at least 90% or more of R39-R43 binding ability. “Weak binding” ability defined peptides with statically less interaction with R39-R43 epitope but having more than 60% of ability of interaction than R39-R43 epitope (Figure 1A, left panel and Table S1). However, streptavidin being known to produce unspecific binding, we used the ratio between aPL and IgG control (B/B0) (Figure 1A, right panel). This representation allows thus to distinguish the real levels of interactions. In regards of these results, although the interactions between peptides and aPL are dependent of polarity of the amino-acid as previously described and confirmed in the present study, it seems that the polarity is not sufficient to explain the entire effects observed. Several other properties of amino acids have also been put forward, such as electrical charge or hydropathy (3, 4). Iverson and colleagues demonstrated that the mutation of glycine 40 or arginine 43 leads to the loss of interaction with most aPL (3). Several other research groups have performed epitope mapping using point mutations of DI, and identified D8, D9, K19, S38, R39, G40, M42, R43 and N56T as residues participating in aPL-DI interactions (3,
Although conformation is very important for aPL-β2GP1 reactivity in relation with cryptic epitope exposure, it is now believed that the epitope on DI of β2GPI is three dimensional rather than linear (5-8). This could explain the functional role of D8, D9, G40, M42, R43, although it still does not resolve the function of K19 or N56T. In our recent study, we considered the polarity specific to each amino acid rather than their charges, and this strategy enabled us to identify a unique motif common to all epitope mutations described up until today (2). For example, lysine is a polar and charged residue while glutamine is polar but uncharged. Consistently, Zager and colleagues demonstrated that epitopes recognized by aPL all contained the φφφζζ part of the motifs (9). Another group also described an aPL binding epitope corresponding to the φφφζζ part of the motifs (10). 44% of the mutation described as critical for aPL binding in DI, i.e D8, D9, K19, S38, R39, G40, M42 and R43, are located inside of φφφζζFxφ motifs. If we include the mutations directly adjacent to φφφζζFxφ, i.e. D8, D9 and K19, 100% of the critical mutation for aPL binding in DI are related to the φφφζζFxφ motif (2).

In this context, other physical properties of amino acids such as hydropathy or steric volume could participate to antigenic specificity of aPL. To confirm this hypothesis and as described above, we have selected 8 peptides with high binding ability (>90 %) (Figure 1A and B, blue bars) for aPL as well as 8 peptides with weak binding ability (≥60 %)(Figure 1A and B, yellow bars). We can observed that steric volume of peptides with high and weak binding ability are very similar for each position of amino-acid (Figure 1c and Table S1). However, high binding peptides have a smaller and bigger steric volume at position 3 and 4, respectively, in comparison of weak binding peptides (Figure 1C and Table S1). In another hand, there is only one position with difference in hydropathy between high and weak binding peptides (Figure 1D). Consistently with the present finding, Iverson and colleagues have indeed shown that M42K and M43V mutations have a significant effect on reactivity with aPL while methionine and valine are nonpolar (3). We examined whether the volume of the amino acids
might also explain these differences, and indeed found that methionine and valine differ in volume (162.9 vs 140 Å³, respectively) (Table S2) (11). Nevertheless, volume does not appear to be as important as polarity. Indeed, as seen in the M42K mutation, the methionine and lysine have similar volumes (162.9 vs 168.6 Å³, respectively) (Table S2), but lysine is polar. It seems that it is polarity that gives lysine the ability to affect the interaction with aPL (Table 2). The ability of mutation M42K to interact with aPL is significantly less than mutation M42V (3). In the same way, the ability of mutation R43G to interact with aPL is consistently less than mutation M42K, after substituting a polar residue with a steric volume of 173.4 Å³ with a nonpolar residue with a steric volume of 60.1 Å³ (Table 2 and S2) (3). On the other hand, mutation N56T supports the potential role played by volume in amino acid interactions with aPL. While asparagine and threonine are indeed both polar with very similar volumes, mutation N56T has only a weak effect on aPL binding (Table 2). As mentioned above, we also considered hydropathy as an additional factor affecting aPL interactions, and found that mutation M42K demonstrates a strong variability in hydropathy between methionine and lysine, while methionine and valine have similar hydrophobicity values (Table 2). This particular property could also explain the weaker level of binding observed for mutation R43G. We can thus observed that mutation R43G carries all the differences of physical properties identified for aPL binding. A last and unique physical property also attributed to glycine and proline is their propensity to change the conformation of a polypeptide. Glycine indeed lacks a side chain, giving it a high degree of flexibility and the ability to adopt conformations which are sterically not possible for other amino acids. Glycine is thus frequently found in turn regions of proteins where the backbone has to make a sharp turn. Similarly, proline possess a secondary amine bestow particular structure inserting a bend on the protein chains and breaks α helices and β sheets (12).
Discussion

Although the aPL properties suggest that it is the structure of motifs rather than a specific sequence of amino acids that confers the strongest ability to interact with epitope, they also suggest that it is the level of intricacy observed in the structure of the epitopes that makes them so difficult to identify. In this context, although is now widely accepted that the β2GP1 is the main antigenic target for aPL, numerous receptors have been suggested for aPL-β2GP1 complex suggesting its polyspecificity: in vivo knock-out mice models have shown that Toll-like receptor (TLR) 4 (13), annexin A2 (14), ApoER2 (15), as well as several complement factors (16) decrease the pathogenic effects of aPL. Ex vivo studies performed in primary monocytes and endothelial cells also suggest a potential function of the aPL receptor on TLR2 (17-20), CD14 (19, 21), GPIbα (22), TLR8 (23), as well as on ApoER2’and GPIbα in platelets (24, 25). Beyond the well-known controversy around the respective contribution of TLR2 and TLR4 in APS, different studies on the topic of endothelial cell activation have led to contradictory conclusions, although not necessarily mutually exclusive. In addition, it has been described that anti-annexin A2 antibodies as well as bivalent anti-annexin A2 (Fab’)2 fragments were able to activate endothelial cells in a similar way to anti-β2GP1. Monomeric anti-annexin A2 Fab fragments inhibit these activations but also those caused by anti-β2GP1 in a concentration-dependent manner (26). Zhang and McCrae have, furthermore, observed a lack of complete inhibition, even when using the monomeric Fab fragment at a concentration approximately 6-fold greater than the anti-β2GP1 antibody (26). In regard of these specific studies and the number of different receptors described for aPL, the question arises as to whether aPL epitopes share a common antigenic determinant common to all potential receptors. Indeed, if we assume that the epitope of aPL is a structural motif in the molecule, its inhibition by a monomeric Fab fragment of activation induced by anti-annexin A2, anti-annexin A2 F(ab’)2 or anti-β2GP1 turns it into a competitive binding site for the same epitope. In a recent
publication by our group, we were indeed able to show that inside the interaction between aPL-epitope, the specific amino acid sequences are less important than are the structural motifs in the molecule (2). Equally, Zhang and McCrae have suggested that the antibodies induce a cross-linking or clustering of annexin A2/β2GP1 complexes on the cell surface, leading to cell activation (26). Based on this concept, we were able to identify a structural motif common to all the different epitopes and receptors described, and further demonstrated that five structural epitopes are present on β2GP1 (2). This could explain why a 6-fold greater concentration of monomeric anti–annexin A2 Fab is required to inhibit activity than of the anti-β2GP1 antibody, especially when annexin A2 carries a β2GP1-like epitope (2). It has also been shown that aPL interact more strongly with TLR2 on monocytes and cytokine activated endothelial cells than with TLR4 (19). TLR4 possess a β2GP1-like epitope while TLR2 contains the same epitope recognized by anti-β2GP1 (2). Several studies have additionally shown that TNF production induced by aPL in monocytes was dependent on TLR7 and TLR8 (23, 27, 28). TLR7, TLR9, TLR6 and TLR1 have the same β2GP1-like epitope as TLR4, whereas TLR8 displays a β2GP1-like epitope similar to that of annexin A2 (2). Bioinformatic analyses have demonstrated that the motif recognized by anti-β2GP1 is present and accessible in 16 different proteins within the whole human proteome. If recognition is extended to β2GP1-like epitopes, these motifs are present in all aPL-associated receptors described in the literature (29). Finally, to our knowledge, β2GP1 has the particular property of being the only protein in the whole human proteome to possess 5 potential accessible epitopes for aPL.

The model of 'lock and key' of antibody-epitope interaction is the dominant dogma in immunology. However, past and recent studies establishing the existence of a large number of monoclonal antibodies that can interact with a variety of totally unrelated self and foreign antigens shake this idea up (30-32). While a loss of B cell tolerance mechanisms could explained the polyspecific BCRs present in some naïve B cells leading to polyreactive
antibodies, their proportion do not exceed 20%. (30, 33). This mechanisms have been described with many others to explain the polyspecificity of these antibodies such as the rigid adaptation, the conformational diversity or the differential ligand positioning (34). However, the fact that an epitope, continuous or discontinuous, is described only as a sequence of amino acid has never been questioning. The classical model of the antibody-protein interactions shows that the structures recognized by the complementarity-determining regions (CDRs) on antibody are surfaces created by amino acids. Thus, a surface complementary between antigen and CDRs determines antigen specificity of the antibody. In this context, based a trained neural network, Mason et al. have constructed a library of rationally designed mutations in CDRH3 of trastuzumab, which targets a trained neural network man epidermal growth factor receptor 2 (HER2), and then screened for retention of binding to the HER2 receptor (35, 36). They have found 30 variants to bind the HER2 antigen with significant affinities although they were unable to derive sequence-based rules explaining the common binding specificities (35, 36). Despite the application of highly sophisticated neural network methods using reliable experimental data to train the networks, they concluded that interaction antibody-antigen cannot be explained basically on inspection of amino acid sequences of the antigen (36). Consistently, very recent research performed by Bradley and Thomas on the CD4+ T-cell receptor ‘ligand’ repertoire have showed that: “Within an epitope-specific repertoire, a portion of the responding receptors cluster closely together based on shared motifs” (37). Taking these observation together, the nature of physical properties defining an epitope should be extended for a better understanding and prediction of antigenic sequences. Indeed, the forces involved in these noncovalent interactions including electrostatic interactions, electric dipoles or short-range van der Waals forces are well known (38). The classical model shows that amino acids through the physicals properties determine the epitope but not that the physicals properties themself define the epitope. This novel paradigm of antigenic determinant highlight the wide diversity of strategies
of immune system to enhance its assortment of antibodies which goes beyond the potential prediction from its primary sequence covering thus a broader part of the antigenic universe.
Methods

Ethics statement

In accordance with the decision of the 7th April 2014 of the Cantonal Research Ethics Committee of the Geneva (CCER@etat.ge.ch), all experimental protocols were approved under the Protocol n° 09-072 entitle: “Pathogenic effects of antiphospholipid antibodies”, and with the Declaration of Helsinki, the blood bank obtained informed consent from the donors, who were informed that part of their blood would be used for research purposes.

Patient characteristics

All patients had an APS as defined by the revised Sapporo criteria (39) and, although have been previously described (2, 20), the laboratory parameters relative to APS patients are presented in Table 1 as well as the clinical manifestations. CRYOcheck™ is used as plasma control (PrecisionBioLogic).

Protein Array

Pierce™ Streptavidin Coated High Capacity Plates 96 well plates (Thermofischer) were coated with 10 μg/ml each peptide/well prior to incubation with aPL or control Plasma. Secondary anti-human IgG antibodies conjugated to IR800 (Rockland, Gilbertsville, PA, USA) were used. Antibody-bound antibodies were detected and quantified by the Odyssey system (Li-Cor Biosciences). The biotinylated peptides libraries were generated by Genscript (Netherlands). Lyophilized non water-soluble peptides were reconstituted in 50% dimethyl sulfoxide (DMSO) and 7.5% acetic acid before dilution in PBS. All peptides had >95% purity as assessed by analytical reverse phase high performance liquid chromatography (RP-HPLC). Solid phase immunoassays have been performed with unmodified plasma from APS patients.

Statistical analysis
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Author Contributions

K.J.B. and F.M. wrote and edited the manuscript. K.J.B. performed the experiments and the data analysis

Authorship

Contribution: K.J.B. design the experiments and interpret the results. K.J.B. and F.M. wrote the manuscript

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### Table 1
Clinical and laboratory profiles of the 11 patients providing the aPLA

| Patients | LA  | aCL IgG (GPL) | aβ2GP1 IgG (U/ml) | Clinical manifestations | Age | Gender |
|----------|-----|---------------|-------------------|------------------------|-----|--------|
| Patient 1 | POSITIVE | 31.7 | 24 | Arterial thrombosis | 24 | F |
| Patient 2 | POSITIVE | 59 | 126 | Thromboembolism | 59 | F |
| Patient 3 | POSITIVE | 57 | 49 | Recurrent fetal loss | 48 | F |
| Patient 4 | POSITIVE | 55 | 82 | Recurrent fetal loss | 52 | F |
| Patient 5 | POSITIVE | 14.9 | 25 | Venous thrombosis | 24 | F |
| Patient 6 | POSITIVE | 60 | 106 | Thromboembolism | 43 | M |
| Patient 7 | POSITIVE | 42.8 | 50 | Recurrent fetal loss | 62 | F |
| Patient 8 | POSITIVE | 60 | 90 | Arterial thrombosis | 61 | F |
| Patient 9 | POSITIVE | 19 | 12 | Thrombosis | 53 | F |
| Patient 10 | POSITIVE | 60 | 110 | Venous thrombosis | 42 | F |
| Patient 11 | POSITIVE | 50 | 56 | Venous thrombosis | 79 | M |

| Median (IQR) | Age | Gender |
|--------------|-----|--------|
| 55 (42.3 – 95.0) | 48 | 81.8 % (F) |
| 41.5 (25.0 – 126.0) | | |

aCL IgG anticardiolipin IgG (normal value < 5GPL), aβ2GP1 IgG (normal value < 17 U/ml); F = Female; M = Male; IQR = interquartile range

### Table 2

| 1Mutations | 1% of aPL Binding | p-Values | Polarity | 2Volume (Å³) | 3Hydropathy |
|------------|-------------------|----------|----------|--------------|-------------|
| M42K       | 70 (28-62)        | 0.0001 (vs wt) | nonpolar | 162.9 | 1.9 | -3.9 |
| M42V       | 85 (70-101)       | 0.0003 (vs wt) | nonpolar | 162.9 | 1.9 | 4.2 |
| R43G       | 42 (28-62)        | 0.0001 (vs wt) | polar | 173.4 | -4.5 | -0.4 |
| G40E       | 77 (63-93)        | 0.0001 (vs wt) | nonpolar | 60.1 | -0.4 | -3.5 |
| N56T       | 90 (63-109)       | 0.0001 (vs wt) | polar | 114.1 | -3.5 | -0.7 |

1From Iverson et al., *J immunol.* 2002; 2From Zamyatin, *Prog. Biophys. Mol. Biol.*, 1972; 3Kyte and Doolittle, *J. Mol. Biol.*, 1982
Figure Legend

Figure 1: Physical properties as antigenic determinant of aPL. (A) Quantification of aPL binding to 44 peptides containing motif $\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phi\phil
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