Antigen Specificity of Antiphospholipid Syndrome-Related Antiphospholipid Antibodies

Ricardo Forastiero*

Department of Physiology, Favaloro University, Division of Hematology, Thrombosis and Haemostasis, University Hospital, Favaloro Foundation, Buenos Aires, Argentina

Abstract: The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of antiphospholipid antibodies (aPL) and clinically by vascular thrombosis and/or recurrent pregnancy morbidity. The APS-associated aPL are mostly directed against β_2-glycoprotein I (β_2GPI) and prothrombin. In contrast, aPL linked to infectious disorders are primarily directed against phospholipids and do not tend to be associated with the clinical manifestations of APS. This distinction, however, was later found not to be absolute. Patients with lepromatous leprosy have a high prevalence of true antibodies to β_2GPI, but they do not develop thromboembolic complications. Current evidence suggests that autoantibodies to β_2GPI from APS patients preferentially bind to critical epitopes in domain I of β_2GP1, and leprosy-related aPL recognizing β_2GPI tend to be directed against domain V. The position of the epitope in the β_2GPI molecule seems to be crucial in defining the pathogenicity of aPL. In addition, an increasing number of other phospholipids-binding proteins with central functions in the regulation of blood coagulation and fibrinolysis are also targeted by APS-related autoantibodies. This review intends to highlight the diverse antigen specificity of aPL and likely further studies would help to understand the different clinical behavior of patients with APS and infections-related aPL.

Keywords: Antiphospholipid antibodies, infections, antiphospholipid syndrome.

INTRODUCTION

By the early 1940s, it was demonstrated that the phospholipid cardiolipin was an important component in an immunologic-based test used for the screening of syphilis. By using widely this assay it was soon observed that there were persons with chronically false positive serologic tests for syphilis. These individuals who do not have syphilis commonly had systemic lupus erythematosus (SLE) or other autoimmune diseases. In addition, SLE was associated with false positive tests for syphilis and the occurrence of a circulating anticoagulant which inhibits phospholipid-dependent coagulation reactions [1, 2]. It was shortly called lupus anticoagulant (LA), although they also occur in individuals without SLE or SLE-like syndromes. In view of this association, Harris *et al.* [3] developed a solid-phase immunoassay with cardiolipin as antigen with the purpose to allow a more sensitive detection of the antibodies determined by means of both the syphilis screening and LA tests. Anticardiolipin antibodies (aCL) were then added as a member of the antiphospholipid antibodies (aPL) family. In the 1980s, in view of the accumulating evidence of an association of some clinical features with the presence of aPL, Harris and Hughes described a new clinical disorder [4]. It was first called the anticardiolipin syndrome and later on the antiphospholipid syndrome (APS).

By using widely the aCL ELISA for the detection of aPL in the diagnosis of the APS, it was quickly noticed that this test is also regularly positive in patients with viral as well as bacterial infections [5]. Syphilis was the first infection to be recognized as being linked to aPL. There are also several reports on the association of aCL with infections such as human immunodeficiency virus (HIV), hepatitis, tuberculosis, leprosy, cytomegalovirus, mycoplasma, Q fever, parvovirus, etc. [6-12]. In most cases aCL disappear after resolution of the infections and the titer is usually low positive. IgM aCL are generally more prevalent than IgG aCL. LA activity is transient, weak and less commonly detected in comparison with aCL. In lepromatous leprosy, however, LA is often detected and its activity is the strongest reported in infectious disorders and very high titers of aCL are found [9]. Since the beginning, several authors have tried to find out differences between APS and infections-related aPL, principally focusing on differences in binding to cardiolipin and other phospholipids, IgG subclasses, affinity, etc.

Preliminary classification criteria for definite APS were formulated in 1998 in Sapporo, Japan [13]. Based on results from prospective studies and on the strongest experimental evidence, there was consensus that only vascular (venous or arterial) thrombosis and/or obstetric complications characterize the clinical criteria for definite APS. Among the laboratory criteria, LA and/or aCL of IgG and/or IgM isotypes at medium or high titers must be present on two or more occasions at least 6 weeks apart to avoid transient aPL generally associated with acute infections. Both assays must be performed using standardized methods and following international guidelines [3, 14]. The Sapporo criteria were recently revised and published as a consensus statement in 2006 [15]. In the original classification criteria as well as the most recent one, APS requires the combination of at least one clinical and one laboratory criterion. The revised APS classification criteria provide clearer definitions for clinical features

*Address correspondence to this author at the Hematología, Universidad Favaloro, Solís 453, (C1078AAI) Buenos Aires, Argentina, Tel: +54 11 4378 1145, Fax: +54 11 4378 1311, E-mail: rforastiero@favaloro.edu.ar

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and suggestions to stratify groups of patients. The evaluation of coexisting inherited and acquired thrombosis risk factors in APS patients is highly recommended. Consequently, patients fulfilling APS criteria must be stratified according to the presence or absence of contributing causes of thrombosis. Among the laboratory requisites, the detection of persistently elevated levels of IgG/IgM aPL on two occasions at least 12 rather than 6 weeks apart was modified. In addition, the criterion of medium or high titers aPL was now specifically defined as titers higher than 40 units or higher than the 99th percentile. Despite advances in standardization, many difficulties remain in the performance of these tests and in the interpretation of results [16-19]. The updated version of the recommendations for LA detection and diagnosis was recently published [20]. The 2006 international consensus statement accepted antibodies to β_2-glycoprotein I (anti-β_2GPI), along with LA and aCL, as diagnostic autoantibodies in APS. β_2GPI as outlined below is the most important antigenic target having a central role in the APS.

KEY ISSUES ABOUT ANTIGEN SPECIFICITY OF aPL

It has been demonstrated that most aPL are not directed against anionic phospholipids, as had previously thought, but are a part of a large family of autoantibodies against phospholipid-binding plasma proteins. In 1990, three groups simultaneously reported that some aCL were not directed to cardiolipin itself, but to the plasma protein β_2GPI which binds to cardiolipin [21-23]. Further evidence demonstrated that LA activity requires either β_2GPI or prothrombin [24, 25]. There is enough support indicating that phospholipid-binding proteins such as β_2GPI and prothrombin are in fact real antigens rather than protein cofactors for aPL [26-28]. Autoimmune aPL requiring phospholipid-binding proteins to bind to phospholipids, and alloimmune or infectious aPL not requiring proteins in their phospholipid binding activity are the main two types of aPL that have been described [19, 29]. Most autoimmune aPL reacting with β_2GPI account for aCL reactivity, whereas those with specificity toward β_2GPI or prothrombin (anti-PT) exhibit LA properties.

The most relevant antigenic targets involved in APS are β_2GPI and prothrombin [29, 30]. Nevertheless, a rising number of other phospholipid-binding proteins with central functions in the regulation of blood coagulation and fibrinolysis are also targeted by APS-related autoantibodies [29, 31]. Ttissue type plasminogen activator, plasminogen, annexin A2 (AnnA2), annexin A5, tissue factor pathway inhibitor (TFPI), protein C, protein S, endothelial protein C receptor, and thrombin, were reported in more than a few papers [32-41]. More limited data propose other potential antigenic targets, including factor XII, factor XI, factor IX, factor VII/activated factor VII, prekallikrein, high and low molecular weight kininogens, vascular heparan sulfate proteoglycan, complement component C4, complement factor H, CD36, protein Z, activated protein C, plasmin, plasminogen activator inhibitor-1 and heparin-platelet factor 4 [42-50]. The importance of this large group of other antigens was analyzed in detail elsewhere [29]. In this review, we present our current knowledge on β_2GPI and prothrombin, the dominant antigenic targets recognized by aPL in patients with APS (Table **1**).

Table 1. Main Antigens Targeted by Antiphospholipid Syndrome (APS)- or Infections-Related Antiphospholipid Antibodies (aPL)

Antigen	Main aPL
β_2-glycoprotein I (domain I)	APS-related
β_2-glycoprotein I (domain V)	Infections-related (leprosy)
Prothrombin	APS-related
Cardiolipin	Infections-related
Proteins involved in blood coagulation and fibrinolysis	APS-related

β_2GPI

β_2GPI is a 50kDa glycoprotein containing five domains termed I through V. Domain V contains a lysine-rich region which is positively charged and then binds to negatively charged phospholipids [51, 52]. The binding of β_2GPI to anionic phospholipids or cell surfaces exposing phospholipids is rather weak in comparison to other proteins such as clotting factors. Recent data have demonstrated that *in vitro* β_2GPI interacts with a number of cell types through surface receptors. Among them, apolipoprotein E receptor 2', AnnA2, glycoprotein (GP) Ibα (GPIbα) subunit of the GPIb/IX/V receptor, and receptors of the Toll-like receptor family [53-55]. Domains II to V of β_2GPI are involved in its interaction with platelet GPIbα. β_2GPI binds weakly to phospholipid surfaces but anti-β_2GPI increase the affinity of β_2GPI for anionic phospholipids 100 times, and thereby decrease the available phospholipid surface for the assembly of coagulation enzyme complexes.

β_2GPI is required for the binding of autoimmune aCL to cardiolipin. The detection of true antibodies to β_2GPI requires the use of suitable negatively-charged or high-binding surfaces [26, 27]. Home-made ELISA, and western and dot blot assays have been proposed to detect anti-β_2GPI [56]. Two theories explain why antibodies to β_2GPI can be detected only on appropriate ELISA plates. In the first hypothesis there is a need of a high density of β_2GPI on the microtiter plate. This is consistent with the observed low affinity of anti-β_2GPI and that the antibody binding to β_2GPI on a suitable surface depends on the marked enhance in avidity provided by engagement of both antigen-binding sites of the antibody to two β_2GPI molecules (dimerization theory) [27, 57, 58]. The second hypothesis is based on the recognition of a cryptic epitope by aPL [26, 59, 60]. It is well-recognized that a conformational change in β_2GPI does occur upon binding to cardiolipin or oxygenated plates. The family of anti-β_2GPI is heterogeneous comprising immunoglobulins which differ in their biological activities, avidity, and target different epitopes on the β_2GPI molecule [61]. During the last years, a variety of potential epitopes have been identified and they seem to spread all over the five domains of the molecule. The most recent studies show convincing evidence that anti-β_2GPI purified from the majority of patients with APS preferentially bind the domain I of β_2GPI [62-66]. Studies on expressed mutants of whole β_2GPI with incorporated single-point mutations within domain I identified the resi-

dues G40 and R43 as being important in conferring binding to aPL [63]. Additionally, Ioannou *et al.* [66], recently acknowledged that the binding of aPL to β_2GPI domain I is complex and likely to involve discontinuous epitopes that include R39 in addition to G40-R43, the domain I-II interlinker, and possibly a pair of aspartic acid residues at positions 8 and 9. All of these discontinuous epitopes are in close proximity within the tertiary structure of domain I.

Anti-β_2GPI are responsible for aCL reactivity and/or LA activity but in some cases antibodies to human β_2GPI are found in APS patients who do not present reactivity against phospholipids in the classical LA and aCL assays.

PROTHROMBIN

Prothrombin is a vitamin K-dependent single-chain glycoprotein of 579 amino acid residues. During its liver biosynthesis, prothrombin undergoes a post-translational carboxylation at the γ-carbon of their 10 glutamic acid residues located in the amino-terminal domain of the molecule (fragment 1). These γ-carboxyglutamic acid (Gla) residues are necessary for calcium binding, which induces a conformational change in the Gla domain required for phospholipid binding. The complex of activated factor X, factor V, calcium and phospholipids activate prothrombin to thrombin in a sequential reaction. Thrombin mainly triggers fibrinogen polymerization into fibrin, but also binds thrombomodulin on the endothelial cells surface and thus activates protein C which then inactivates activated factor V and VIII. Besides, it is involved in activation of other clotting factors, platelets and endothelium. Anti-PT strongly increase the interaction of prothrombin with anionic phospholipid surfaces and activated platelets [67]. As a result of the increased surface concentration of prothrombin there is an enhancement of thrombin production [68].

In 1959, Loeliger [69] was the first to describe the so-called LA cofactor phenomenon. As low plasma concentration of prothrombin was also found, prothrombin was suggested as the cofactor related to the expression of LA activity. The presence of antibodies reacting with prothrombin in patients with LA and severe prothrombin deficiency was demonstrated in the past by Bajaj *et al.* [70]. They also suggested that anti-PT bind to prothrombin in the circulation leading to the formation and enhanced clearance of prothrombin-containing complexes. This kind of anti-PT shows high affinity for prothrombin and has been associated with acquired hypoprothrombinemia and bleeding in a small subset of patients with autoimmune aPL [71]. However, the majority of patients with anti-PT and LA activity have normal prothrombin levels [72]. In the early 1990s, some relevant studies underlined the role of anti-PT in LA activity [25]. The mode of presentation of prothrombin in solid phase influences its recognition by anti-PT. In fact, prothrombin coated onto gamma-irradiated plates or exposed to immobilized anionic phospholipids such as phosphatidylserine is needed to make possible the binding of anti-PT [28, 73]. Anti-PT are mainly antibodies of low-affinity which bind bivalently to immobilized prothrombin. However, the recognition of cryptic epitopes when prothrombin binds to anionic phospholipids or proper plates cannot be excluded. Rao *et al.* [67] have demonstrated binding of anti-PT to fragment 1 which contains the phospholipid-binding site. By means of recombinant prothrombin fragments coated onto irradiated plates it was shown the presence of two types of anti-PT [74]. Some are directed against fragment 1 and the others against prethrombin 1 (fragment 2 plus thrombin). The largest part of anti-PT are functional causing LA activity but others are non-functional because they do not display LA and can be only detected by ELISA [75-77].

CLINICAL ROLE OF ANTIBODIES TO B_2GPI AND PROTHROMBIN

Since their first report, the relationship between the major clinical features of the APS and anti-β_2GPI or anti-PT has been amply studied. Most of the authors agree that anti-β_2GPI are strongly associated with thrombosis and other clinical features of the APS [33-35, 78-80]. In our first studies including a large population of aPL patients we found a close association between IgG anti-β_2GPI and venous thrombosis but no association with arterial events [81, 82]. According to many retrospective and case-control studies, the anti-β_2GPI assay shows higher specificity than aCL in the recognition of patients with APS. In general IgG is the only isotype significantly associated with a history of venous thromboembolism. Conversely, less consistent results are available about the association of IgM anti-β_2GPI and thrombotic events. Even more, it has been demonstrated that the IgG subclass is important being IgG_2 mainly found in APS [80]. Data on the clinical association of anti-PT are more conflicting. Some studies have shown that anti-PT IgG are significant markers associated with deep venous thrombosis/pulmonary embolism [83-86], while others have found no significant correlation [82]. The clinical significance of anti-phosphatidylserine/prothrombin antibodies was assessed in some studies [87-89]. Anti-phosphatidylserine/prothrombin antibodies were more closely associated with LA and clinical manifestations of APS than were anti-PT. High levels of anti-PT were also found to confer a high risk of myocardial infarction in dyslipidemic middle-aged men without autoimmune diseases [90]. Regarding recurrence of thrombotic events, there are some data suggesting that anti-PT but not anti-β_2GPI are likely a risk factor of recurrent venous thromboembolism [91]. The committee on aPL considered that the inclusion of anti-PT in the classification criteria for APS is still premature [15].

To date, there are many contradictory results on the clinical significance of anti-β_2GPI and anti-PT in pregnancy loss. Some studies, including ours, have shown an association between anti-β_2GPI of IgM isotype and recurrent abortion [92, 93] as well as *in vitro* fertilization implantation failure [93]. Other study including 195 women with two or more unexplained consecutive miscarriages demonstrated that LA and IgG anti-β_2GPI were associated with pregnancy loss but only LA was related to second trimester fetal loss [94]. In contrast, a number of studies showed no association between anti-β_2GPI and/or anti-PT and pregnancy loss or reproductive failure [34, 95-99]. Besides, testing for anti-β_2GPI seems not to identify additional patients with spontaneous abortion or unexplained fetal death who initially have negative results on LA or aCL assays [97, 98]. A large case-control study including 743 women who miscarried in weeks 8 and 9 and 743 women who underwent a first provoked abortion showed that IgG and/or IgM anti-β_2GPI were not independently associated with a first episode of early spontaneous

pregnancy loss [100]. In a study of 170 female patients with primary or secondary APS and a history of pregnancy loss the most prominent result was the significant association of the presence of IgG anti-PT with early pregnancy loss [101]. Large-scale studies are needed in order to draw definite conclusions. According to the available information, there is some debate regarding the exclusion of aPL of the IgM isotype from the laboratory APS criteria [15, 16]. A recent prospective longitudinal study carried out in Europe suggests that laboratory criteria for the obstetrical APS could not be the same for pure obstetrical APS than thrombotic APS [102]. In the European cohort of 109 women having APS, almost half of the women have isolated aPL, with a predominance of aCL.

In a systematic review of the literature, Galli *et al.* [103] did not reach any firm conclusions as regards the role of anti-β_2GPI and anti-PT and the risk of thrombosis. This could be due to the retrospective design that the majority of available studies about the clinical relevance of anti-β_2GPI and anti-PT indeed have [104]. In a prospective cohort study of 194 consecutive unselected patients with persistent classical aPL (LA and/or aCL), we evaluated anti-β_2GPI and anti-PT as risk factors for first or recurrent thromboembolic events [105]. Our data showed for the first time that the presence of anti-β_2GPI and/or anti-PT is a predictor of thrombosis in aPL patients. Our study also identified the male sex and previous thrombosis as major independent predictors of thrombosis during a median follow-up of 45 months. The occurrence of first or recurrent thrombotic events in our prospective study was similar to those reported in other report [106]. For clinical purposes, it is important to find which aPL markers has the best prognostic value. In our study, among patients with LA and/or aCL, the group of patients with anti-β_2GPI and/or anti-PT had an increased risk of first or recurrent thrombotic event. When LA and/or aCL positivity was not associated with antibodies to prothrombin and β_2GPI, the thrombotic risk was lower. The results of a 15-year longitudinal study show that IgG anti-PT is the most useful predictor of thrombosis in SLE patients [107]. An important observation reported by several recent studies is that the risk of thrombosis progressively increases with the number of positive antiphospholipid antibody tests. The triple positivity of LA, aCL and anti-β_2GPI conferred the highest risk of first and recurrent thrombosis [105, 108, 109]. This remark strongly supports the subclassification proposed by the revised criteria according to the positivity for multiple aPL or for a single aPL for patients' enrolment in clinical studies.

In most infections, autoantibodies against β_2GPI and prothrombin are rarely found compared with patients with APS or other autoimmune disorders. Several years ago, we and others have confirmed that no reactivity against phospholipid-free β_2GPI measured by ELISA is detected in sera from syphilitic patients [110, 111]. Thus, it was suggested that anti-β_2GPI assay could clearly differentiate between APS and infections-related aPL. In a series of studies, in patients with other infections such as tuberculosis, Q-fever, Mediterranean spotted fever, Klebsiella, mononucleosis or hepatitis C virus infections similar data were found with a low prevalence of anti-β_2GPI [5, 6, 8, 12]. There are also several reports in HIV infection showing a very low prevalence of anti-β_2GPI as well as anti-PT [7,112]. The belief that infections-related aPL do not bind β_2GPI in the absence of phospholipids has been challenged by recent studies. In an early report, it was reported that in leprosy patients aCL were heterogeneous with respect to their β_2GPI requirement [10]. In half of the patients, aCL were β_2GPI-dependent for binding to cardiolipin. In a series of experiments by our group, in 51 samples from leprosy patients, 29 samples had anti-β_2GPI and 23 had anti-PT [9]. Almost all anti-β_2GPI were of the IgM isotype and titers were in fact very high. Leprosy-related aPL resemble those found in APS but differ largely in the immunoglobulin isotype. Arvieux *et al.* [113] corroborated in lepromatous leprosy the high occurrence of anti-β_2GPI and anti-PT. Besides, IgG_3 was the most common subclass reactive to both β_2GPI and prothrombin in selected high-titer leprosy sera, unlike autoantibodies from APS patients largely restricted to IgG_2. Furthermore, anti-β_2GPI from leprosy showed relatively high avidity binding to fluid-phase β_2GPI, and ability to recognize epitopes located in different domains of the β_2GPI molecule, thereby differing from those found in APS which are mainly directed against domain I. A recent publication reports that anti-β_2GPI are also frequently present in leptospirosis and Kala-azar [11]. In B19 parvovirus infection, aCL were also shown to be β_2GPI dependent and behaved in a similar fashion as leprosy aPL. Overall these findings signify that the distinction between autoimmune (APS-associated) and infectious (β_2GPI and prothrombin-independent) aPL is not absolute.

We also showed that antibodies against TFPI are rarely found in sera from patients with infections-related aPL, even in leprosy patients who have high titers of antibodies to β_2GPI and prothrombin [114]. Antibodies reacting to complexes of heparin-PF4 were frequently detected in sera from patients with autoimmune aPL and leprosy, even in patients who never received heparin treatment. In syphilis and HIV infection, however, these antibodies were mainly not detected [115].

CONCLUSIONS

Over the last years, our understanding of the aPL has made considerable progresses mainly in the area of the different antigens targeted by autoimmune aPL. In addition, significant knowledge has been gained on the nature of the cellular receptors and its interaction with β_2GPI and specific antibodies. Our understanding of the pathophysiology of the APS has consequently greatly improved. For clinical purposes, it is imperative to find which aPL markers has the best prognostic value. In this chapter, we have reviewed the current information on differences between aPL linked to the APS and those related to infection disorders. In the near future it is expected that we will be capable to better distinguish pathogenic from nonpathogenic aPL and therefore to choose the most proper aPL tests for the diagnosis of APS.

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