Avidity of antiphospholipid antibodies – our current knowledge

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ABSTRACT

Antiphospholipid antibodies (APL), which represent serum markers of the antiphospholipid syndrome, comprise an extremely heterogeneous group of autoantibodies directed against various phospholipids and protein cofactors. The heterogeneity of APL includes not only their antigen-binding site specificity but also their avidity. The aim of this study was to summarize the current knowledge about commonly-used procedures for the avidity determination with a special interest in the antiphospholipid antibodies and to evaluate the clinical significance of APL avidity determination. The common techniques in clinical laboratories for avidity determination utilize the ELISAs in the presence of various chaotropic agents. The findings of clinical studies suggest that the high avidity APL are associated with thrombosis and antiphospholipid syndrom (APS). The determination of APL avidity might be a complementary laboratory marker applicable in the classification of APS.

KEYWORDS

antibodies against ß2-glycoprotein I – anticardiolipin antipody – antiphospholipid antipody – avidity – chaotropic agent – ELISA

SOUHRN

Fialová L.: Avidita antifosfolipidových protilátek – naše současné znalosti

Antifosfolipidové protilátky (APL), které představují sérový marker antifosfolipidového syndromu, zahrnují mimořádně heterogenní skupinu protilátek namířených proti různým fosfolipidům a proteinovým kofaktorům. Heterogenita APL se týká nejen specifity vazebných míst, ale také jejich avidity. Cílem studie bylo shrnout dosavadní znalosti o běžně užívaných postupech pro stanovení avidity se speciálním zaměřením na APL a zhodnocení jejího klinického významu. Často užívanou technikou pro stanovení avidity protilátek jsou ELISA metody v přítomnosti různých chaotropních látek. Nálezy klinických studií ukazují, že APL o vysoké aviditě se vyskytují u pacientů s trombózou a antifosfolipidovým syndromem (APS). Vyšetření avidity APL by mohlo být doplňujícím laboratorním ukazatelem, který by přinesl informaci využitelnou pro klasifikaci APS.

KLÍČOVÁ SLOVA

protilátky proti ß2-glykoproteinu I – antikardiolipinové protilátky – antifosfolipidové protilátky – avidita – chaotropní látky – ELISA

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INTRODUCTION

Antiphospholipid antibodies (APL) comprise an extremely heterogeneous group of autoantibodies directed against various phospholipids and protein cofactors. APL represent serum markers of the antiphospholipid syndrome (APS, otherwise Hughes syndrome) whose principal manifestations are venous or arterial thromboses, various obstetric complications and occasional thrombocytopenia [1, 2].

The following antiphospholipid antibodies are included in the laboratory criteria for the diagnosis of APS – anticardiolipin antibodies (ACL), antibodies against β2-glycoprotein I (anti-β2GPI) and lupus anticoagulant. All three antibodies may be detected in some patients with APS, but only one positive APL together with at least one of the clinical criteria are required for the diagnosis of APS [3]. Next to APS, antiphospholipid antibodies are present in the serum of patients with other autoimmune diseases, various infections or malignancies [4–6]. To exclude the possibility of a transient elevation due to the infection-associated antibodies, the positivity of APL must be found repeatedly at least 12 weeks apart after the initial determination for the confirmation of APS diagnosis [3]. The positive ACL are present in 84–90% of APS patients in comparison with only 12–30% patients with systemic lupus erythematosus (SLE). Similar positivity in APS patients was observed for anti-β2GPI antibodies (60–90%) [7].

Although the new so-called Sydney clinical criteria for APS improved the classification of APS, some problems remained to be resolved. All APL used for the classification of APS have certain methodological and diagnostics shortcomings [8, 9]. Anti-β2GPI antibodies are strongly associated with clinical manifestations of APS but they are characterized by quite low sensitivity. Unfortunately, the anti-β2GPI antibody ELISAs detect all antibodies reactive with β2GPI, including nonpathogenic antibodies and phospholipid-independent

anti-β2GPI antibodies, which makes them less suitable as a general diagnostic test. However, a great advantage of anti-β2GPI antibody ELISA tests is the usage of a single and well-defined glycoprotein for coating of microtitrate plates. The diagnostic significance of ACL is currently judged predominantly because of the problems with standardization. Nevertheless, certain limitations of ACL assays, the measurement of ACL by standardized ELISA constitutes one of Sydney's laboratory criteria by virtue of their high diagnostic sensitivity. The specificity of the ACL assay is increased by addition of β2GPI to cardiolipin. Moreover only medium or high levels of ACL antibodies are included as a laboratory criterion. The assays for ACL are considered an additional diagnostic tool [8].

Not all subjects with increased APL antibodies show clinical symptoms. APL are found even in healthy persons [10]. When the cut-off for the ELISA-tests are set at the 99th percentile as stipulated by the Sydney criteria, by definition 1% of healthy individuals will be positive in the test [3]. The isolated APL positivity need not be associated with thromboembolic events and it is not sufficient to make the diagnosis of APS [11]. It may be apparently caused by the heterogeneity of APL regarding their antigen-binding site specificity and their avidity [12].

It is known that the humoral immune response to a specific antigen includes both the quantitative aspect determined as the concentration of immunoglobulins and the qualitative view point of sensitivity, cross-reactivity or specificity. The latter parameter is a function of avidity [13]. The absence of clinical manifestations in APL positive patients may be attributed to the presence of low-avidity APL. So that not only titre, immunoglobulin isotope or antigenic specificity, but also binding affinity may affect the eventual predictive value of APL [14]. The antibodies with higher avidity were shown to identify autoantibodies with stronger predictive value for clinical manifestations. In order to improve the diagnostic significance of APL, some investigators focused on avidity determination. Previous studies suggested the benefit of APL avidity determination for clinical purposes [15, 16].

TERMS OF AFFINITY AND AVIDITY

Affinity describes the strength of binding of the single immunoglobulin paratope with the corresponding antigen epitope [17, 18]. It is produced by the summation of attractive forces that increase binding strength and repulsive forces, which decrease the binding strength. The intermolecular forces present between the paratope and epitope include relatively weak non-covalent interactions such as hydrogen bonds, electrostatic forces, hydrophobic forces or van der Waals forces. Affinity can be expressed as an association constant obtained by the equation derived from the law of mass action [17].

However, naturally occurring immunoglobulin molecules have at least two antigen-binding sites capable to react with multiple antigenic epitopes. The total strength of binding between a multivalent antibody and a multivalent antigen is called avidity or functional affinity [18, 19]. It depends on the affinities of the individual immunoglobulin binding sites for the appropriate epitopes of the antigen as well as on their density, spacing and antibody polyreactivity.

Affinity is an important feature of antibodies that is multi-genetically determined independently on the antibody levels [20]. Generally, affinity of IgG is initially low after primary infection or primary antigenic challenge. Antibodies of increasingly higher affinity become abundant during an immune response as a result of somatic hypermutation followed by antigen driven proliferation of selected clones of memory B cells. Therefore the immunoglobulins produced in a secondary response have higher average avidity than those produced in the primary response. Analysis of IgG avidity is a complementary test that can differentiate the primary and secondary antigen exposure in various infectious diseases such as rubella, toxoplasmosis and others [21–25].

Unlike infectious disease, the differences between primary and secondary immune responses are not clearly expressed in autoimmune disorders and the question of affinity maturation in autoimmune disorders has not been solved [17]. If the antibody is an autoantibody directed against a self-component, and if this antibody is pathogenic, then affinity maturation may be injurious [26].

The determination of autoantibody avidity showed that their different avidity might contribute to the various clinical presentation [26]. It was suggested that high-avidity autoantibodies play a significant role in the organ-specific autoimmune diseases, while the low-avidity antibodies as well as high-avidity antibodies may contribute to the non-organ specific immune complex mediated diseases [18].

Autoantibody avidity is supposed to be low at the beginning of diseases, and then it increases, owing to avidity maturation during the disease course [27]. However, an avidity maturation of autoantibodies may have been completed by the time that patients were presented with the autoimmune diseases [26, 28]. The antibody maturation associated with progression from low to high avidity is not always uniform and may be influenced by many factors [23]. It may vary considerably among individual subjects. It has been shown for the anti-citrullinated protein antibodies in the patients with rheumatoid arthritis [28]. While most of the patients in the predisease stage displayed limited avidity maturation, a small group underwent substantial maturation of avidity.

METHODS FOR ANTIBODY AVIDITY DETERMINATION

Various methods for avidity/affinity determination have been described. The usual techniques in clinical laboratories utilize the solid-phase immune assays in the presence of chaotropic agents [19, 29–31]. The avidity is usually measured by an enzyme-linked immunosorbent assay (ELISA) in which chaotropes disrupt antigen-antibody interactions. This setting of ELISA does not permit measurement of the true equilibrium dissociation constant since antigens and antibodies are in separate phases [16]. As a rule, the initial binding of antibodies to the appropriate antigen under physiologic salt concentration is compared to the binding under more chaotropic conditions in simultaneous analyses [16]. The relative avidity is evaluated in this way. The results of ELISA avidity assays correspond well with those obtained via more accurate measurement using, for example, a biospecific interaction analysis or an equilibrium dialysis [32, 33].

Two procedures have been well described in the literature. In the so-called preventive principle the chaotropic agents are added into the serum diluent to prevent the binding of low-avidity antibodies to the solid-phase antigens (diluting principle) [34]. The other dissociation principle is commonly applied. This procedure includes an extra step after the formation of the immune complexes of specific antibodies and antigens coated on the microtitrate wells. The immune complexes are temporarily exposed to the action of chaotropic agents present in the washing buffer (eluting principle) during the extra step. The interactions of low-avidity antibodies with antigens are easily broken by chaotropic agents, while high-avidity antibodies remain bound to antigens [31, 35].

The released low-avidity antibodies are eluated from the wells before quantification of antibodies bound to the antigens. This method is sometimes denoted as "bind and break" ELISA [36]. Generally, the value of antibody avidity is expressed as an avidity index (AI) whose calculation is based on the ratio or percentage of bound antibodies in the microtitrate wells in the presence or absence of the chaotrope.

This basic methodological approach may be modified in numerous ways. The elution of antigen-antibody complexes has been performed on exposure to various dissociation agents for different lengths of incubation. Distinct chaotropic agents have different abilities in dissociation of immune complexes [29]. Chaotropic agents such as urea, ammonium thiocyanate, sodium chloride or guanidine hydrochloride facilitate dissociation of immune complexes in ELISAs [16, 36, 37]. The intensity in an interruption of antibody-antigen bindings seems to be highly dependent on the kind of examined antigen and its specific antibodies.

Other modifications for ELISA avidity assays consist of a different dilution of analysed antibodies or concentration of chaotropic agents [38]. One approach is based on the determination of avidity on the condition of several antibody dilutions (antibody titre) in the constant concentration of the chaotropic agent [36, 39]. Alternatively, the single diluted serum is exposed to an increasing concentration of the chaotrope [40, 41].

The ELISA assays using chaotropic agents were also applied in the clinical studies focused on APL avidity. Urea or sodium chloride was used as chaotropes [27, 41]. In the case of urea used as a chaotropic agent, the sera were simultaneously tested in serial dilutions with and without urea presence. The term "residual activity" was introduced. It is defined as the serum dilution after urea treatment expressed as a percentage of the serum dilution without treatment and corresponding to the same absorbance [27]. Higher residual activity means the higher avidity of ACL. Čučnik et al. [16, 41] analysed the sera in the presence of increasing NaCl concentration (0.15, 0.25, 0.5, 1, 2, 4 and 6 mol/L). Discrimination between high and low avidity anti-β2GPI antibodies was made arbitrarily by comparing the initial binding of antibodies at 0.15 mol/L with binding at 0.5 mol/L NaCl. When the binding of antibodies at 0.5 mol/L NaCl remained higher than 65% of the initial one, high avidity anti-β2GPI antibodies were declared. When the binding at 0.5 mol/L NaCl decreased to or below 25% of the initial binding, low avidity antibodies were established. The antibodies which did not fulfil these criteria were considered to be of heterogeneous avidity [16].

The most accurate procedures analyse the biospecific interaction between epitopes and paratopes using an equilibrium dialysis or very sophisticated physical method of surface plasmon resonance (SPR) [17, 42]. The method of equilibrium dialysis was the first reliable procedure for estimation of antibody affinity. Unfortunately, the usage of equilibrium dialysis is limited to diffusible small antigens or haptens. SRP is highly sensitive technique which makes possible to study both association and dissociation kinetics of antigen-antibody interactions. However, this technique is an expensive and it is not commonly available.

AVIDITY OF ANTIPHOSPHOLIPID ANTIBODIES

Binding properties of APL with different avidity

Binding characteristics of APL with different avidity were predominantly studied in anti-β2GPI antibodies with respect to the antigen density and its conformational changes. Early experimental studies using ELISA, immunoblotting or affinity chromatography demonstrated that polyclonal anti-β2GPI antibodies are intrinsically mainly of low-affinity, monoreactive antibodies directed to an epitope on native β2-glycoprotein I and that the high density of antigen is necessary for the binding of low-avidity antibodies, which require bivalent interactions with the epitopes of the antigen [43, 44]. Čučnik et al. [45], suggested that neither high density of the antigen nor high avidity of the antibodies (or Fab fragments) alone was sufficient for the binding of anti-β2-GPI antibodies to β2-GPI. The density of the antigen is not so important for the high-avidity antibodies characterized predominantly by monovalent bindings. They supposed that some conformational modifications and, consequently, exposed neo-epitopes were required for the recognition of β2GPI by polyclonal anti-β2GPI antibodies.

Recently, the analysis of anti-β2GPI antibodies using surface plasmon resonance confirmed that high-avidity antibodies interact predominantly monovalently with much lower dependency on the antigenic density and that they form more stable bimolecular immune complexes than those with low-avidity [120]. Sheng et al. [43] proposed a model in which β2GPI, acting as an Ag in vivo, is bound by anti-β2-GPI antibodies only when clustering of β2-glycoprotein I on membrane surfaces, such as endothelial cells, occurs. These conditions may induce endothelial cell activation. Actual conformation of β2-GPI can also influence the binding properties of anti-β2-glycoprotein I antibodies. While low-avidity antibodies recognized only the open conformation of β2-GPI, the high-avidity antibodies are capable of interacting with the circular conformation present in plasma [46].

In this context the testing of more appropriate solid phases applicable for APL assay techniques seems to be important. Membrane surfaces like hydrophobic polyvinylidenedifluoride (PVDF) used in immunodot methods make possible a denser presentation of hydrophilic part of phospholipids on the membrane. It facilitates the bivalent binding which is required for medium and low affinity APL [47].

Clinical usefulness of APL avidity determination

The existing clinical studies had predominantly evaluated the avidity of IgG isotype of APL. The determination of APL avidity had been usually performed in a solid phase in most of the clinical studies. Mainly urea and NaCl were applied for the disruption of bindings between antigens and antibodies. Despite different experimental conditions, the results did not markedly vary. The early clinical studies on APL avidity used urea for the dissociation of immune complexes [48]. They established that antibodies to either β2-glycoprotein I or cardiolipin/β2-glycoprotein I complex derived from APS patients present a high resistance to urea in contrast to the sera of non-APS patients with autoimmune disease. Celli et al. [49] observed high-avidity APL in 30% of patients with APS and SLE. Patients with syphilis presented uniformly low-avidity APL similarly as those with others infectious disorders like malaria or acquired immunodeficiency syndrome [50]. APL, which did not recognize β2-GPI and, occurring in HIV-1 infection, were also of low resistance to dissociating agents (urea or NaCl) [51]. Within this context, the observation of high-avidity anti-β2GPI antibodies in the patients with leprosy but not in those with APS was surprising [52] and perhaps was explained by the different methodology of avidity determination.

As in previous studies, Čučnik et al. [41], who used NaCl in several increasing concentrations for avidity determination, suggested that high-avidity anti-β2-GPI antibodies are clinically more relevant than low-avidity ones. The presence

of high-avidity anti-β2-glycoprotein I was not rare in the patients with APS. Thrombosis predominantly venous was the main clinical feature associated with high-avidity anti-β2-glycoprotein I antibodies, while low-avidity anti-β2-GPI antibodies may prevail in pure SLE. Thrombosis was rare in the group of patients with low-avidity anti-β2GPI even though they had higher titres. De Laat et al. [53] also found that anti-β2-GPI antibodies with high avidity better correlate with thrombosis than those with low-avidity. The anti-β2--GPI antibody avidity seems to be a rather stable parameter in an individual patient. Čučnik et al. [15] assume that avidity of anti-β2GPI may be a more reliable laboratory feature than APL titre for the evaluation of long-term thrombotic risk.

The promising preliminary results inspired the experts to design the multicentre studies that confirmed the published data [16, 54]. Additionally to the previous findings, this recent, further extended, multicentre study of the same investigation team also clarified a clear association between high-avidity anti-β2-GPI and obstetric complications. Statistically significant difference was observed in patients with obstetric disorders in the group of high avidity antibodies versus low avidity.

Moreover, high-avidity ACL comparable to those in APS patients were reported in patients with primary biliary cirrhosis, primary sclerosing cholangitis and type 1 autoimmune hepatitis [27, 39]. These findings may imply the possibility of developing APS features during the course of autoimmune liver diseases.

Surface plasmon resonance was utilized for the comparison of binding kinetics and affinities of APL in patients with APS, patients with a positive VLDR (Venereal Disease Research Laboratory) test and healthy subjects. The evaluation of the binding curve was applied for the discrimination of patients with APS and syphilis. The ACL found in the APS patients showed a higher association rate, while the ascending dissociation curve of the ACL from VLDR positive sera point to rebinding phenomena often seen in low affine IgM antibodies [55]. The used biosensor was able to detect the ACL in the APS patients with higher sensitivity than routine ELISA and additionally allowed the analysis of binding kinetics and affinities.

In conclusion, the findings of clinical studies suggested that avidity of APL may be clinically useful. The high-avidity APL are associated with thrombosis predominantly venous and APS. It seems that avidity could be a valuable additional characteristic of APL whose determination might contribute to the classification of APS. The modified ELISA method, using various chaotropic agents for dissociation of immune complexes, is a suitable method for routine avidity determination; however, the results obtained by plasmon surface resonance are characterized by higher sensitivity.

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