Anti-idiotypic regulation of cofactor--independent antiphospholipid antibodies

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Abstract

Background: *Antiphospholipid antibodies (APA) response development and regulation is still unclear, therefore APA clinical role or association is still on debate. In our previous study the difference in APA specificity and cofactor-dependency in patients with autoimmune and non autoimmune diseases was shown. In this investigation we studied APA-antiidiotypic antibodies (Abs) network in chronic viral hepatitis B, systemic lupus erythematosus (SLE) patients and in women undergoing in vitro fertilization (IVF).*

Material and methods: *Sera of 32 patients with SLE, 30 with chronic viral hepatitis B, 142 women undergoing IVF and 82 healthy individuals were inspected for APA and anti-idiotypic antiphospholipid antibodies (AiAPA). Anti-idiotypic antiphospholipid antibodies were examined by two different methods: 1) direct binding AiAPA in ELISA with mouse monoclonal cofactor-independent APA (mAPA); 2) antiidiotypic neutralization of monoclonal APA reaction with phospholipids; antiphospholipid antibodies positive women undergoing IVF were treated by low dose intravenous immunoglobulin (IvIg), their serum was tested for APA and AiAPA levels before and 5 day after treatment.*

Results: *In APA+ women undergoing IVF the mean level of AiAPA was significant lower than in APA- women in the same group ($p < 0.05$). Intravenous immunoglobulin (IvIg) infusion authentically reduced antiphospholipid antibodies as well as raised AiAPA levels in APA+ women undergoing IVF.*

Conclusion: *Result of this research confirmed a natural AiAPA participation in regulation of production cofactor-independent APA.*

Key words: *anti-idiotypic regulation, cofactor-independent antiphospholipid antibodies, IvIg therapy.*

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Introduction

Antiphospholipid antibodies (APA) are a highly heterogeneous family of autoantibodies. They vary in phospholipids specificity, cofactor-dependency and as a result in implication and/or associations with different clinical manifestation. β2-glycoprotein-I dependent (β2GP1) APA are reliable diagnostic markers of antiphospholipid syndrome (APS) [1] and strongly associated with the occurrence of thrombotic events [2, 3]. However, it was reported that β2-GP I antibodies have been closely associated with venous thrombosis but not with other clinical features of APS, such as arterial thrombosis and recurrent abortion [4].

β2GP1-independent APA are described in patients with infection, AIDS, syphilis [5], viral hepatitis [6], malaria [7], and in women with reproductive failures [8]. The clinical relevance of antiphospholipid antibodies in women with reproductive failure is controversial [9-12]. β2GP1-independent APA are generally not associated with thrombotic incidence [13] but can exert lupus anticoagulant activity *in vitro* [14]. Antiphospholipid antibodies associations with pregnancy outcome and *in-vitro* fertilization (IVF) failure have recently been reported [10]. At the same time a lot of authors disclaim this association [12, 15]. However, in most of these reports, APA specificity distribution and cofactor dependence were not investigated.

As a matter of fact, any hypothesis, which links APA with specific clinical implication, has to consider such antibodies presence in patients with infection, tumors, and what is most important, among healthy individuals [16, 17].

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The nature of anti-phospholipid antibodies as well as regulation and development of APA response is the essence of intensive investigation.

In the 1970s Jerne [18] proposed the network hypothesis, in which complementary interactions involving idiotypes and antiidiotypes were suggested to contribute to the homeostasis of the adaptive immune response. Most its experimental validations network theory vindicated in studies of antibody response against non-protein antigens [19, 20]. The idiotypic/antiidiotypic network was, in the past, a field of intensive research in autoimmunity. It has been shown that antiidiotypic antibodies can act as regulators of the autoimmune response in systemic lupus erythematosus (SLE) [21]. The detection of antiidiotypic antibodies in clinical specimens is challenging, since most autoimmune diseases involve polyclonal responses to self antigen. Data for prevention of experimental APS development in mice by monoclonal AiAPA [20, 22], and also effective treatment of APA positive patients by intravenous immunoglobulins (IvIg) [23, 24] support the idea about anti-idiotypes interaction as the regulation link in APA production.

In our previous study the difference in APA specificity in patients with autoimmune and non autoimmune diseases was shown [25]. We showed that APA in IVF women and in children with chronic viral hepatitis B (CVHB) are mostly β2GP1-independent as compared with APA in SLE patients. Similar results were published by Biron and Guglielmone, who found that a high proportion of patients with HCV [26] and other viral infections [27] and positive aCLs were cofactor independent.

In this investigation we studied APA-AiAPA network in serum of patients with CVHB, SLE and in women undergoing IVF. We also investigated single IvIg infusion effect on APA-AiAPA system in IVF patients.

Material and methods

Human sera

Serum samples from 32 patients with SLE, 30 with chronic viral hepatitis B (CVHB), 142 women undergoing *in vitro* fertilization (IVF) and 82 healthy individuals were examined. Eighteen APA positive women from IVF group were treated by low-dosing IvIg (once 60-80 mg/kg) Biovein (Biofarma, Kiev, Ukraine). In these cases serum samples were taken just prior to the infusion (sample A) and 5 days after manipulation (sample B). Serum of 11 APA positive women was taken 5 days apart due to control. All the subjects had signed an informed consent prior to joining the study.

Monoclonal antibodies

Mouse monoclonal APA (mAPA510) was prepared in our laboratory and defined as cofactor-independent aCL/aPS IgM λ [28]. We used irrelevant mouse monoclonal anti-hTNFα IgMλ (1C1) as a control Ab.

Measurement of APA antibodies

The APA ELISA was performed according to our previous publication [28] and was sensitive to β2GP1-independent and β2GP1-dependent APA. Ninety-six well-polystyrene plate (polySorp, Nunc, Roskilde, Denmark) were covered within 30 µl/well of 50 µg/ml cardiolipin (CL) or phosphatidyl serine (PS) solution (all reagents from Sigma-Aldrich, Steinheim, Germany) in ethanol or ethanol alone (as control well). After that, wells were blocked by 0.5% BSA in 0.05 M Trizma buffer pH-8.5. Serum samples were instilled to the well for an hour after diluting 1 : 50 in RPMI medium with 0.3% BSA, 0.01% Tween 20. The amount of bounded aPL Ab was found out by incubation with horseradish peroxidase-conjugated goat antihuman IgG λ chain specific and reaction of peroxidase with TMB. Optical density (OD) was read at λ = 450 nm using a Multiscan (LabSystems, Finland). Values above 10 GPL for CL and 10 U/ml for PS were considered as positive.

Measurement of antiidiotypic antibodies in human sera

Mouse monoclonal APA (mAPA510) was used as the target Ag in anti-idiotype binding ELISA. We used the same isotype mAb 1C1 as negative control. Polystyrene plates (maxiSorp, Nunc) were coated within mAPA510 (or mAb 1C1 control well) in concentration 5 µg/ml in PBS. After that wells were blocked by 1% BSA in PBS. Serum samples were applied to the well after diluting 1 : 50 in PBS with 0.5% BSA, 0.05% Tween 20 or as a specificity control in the same diluents, A – containing 20 µg/ml mAPA510 or B – 20 µg/ml mAb 1C1. After 2 h incubation plates were washed and reaction was visualized as in previous methods. Anti-idiotypic antiphospholipid antibodies levels were calculated = (OD sample – OD control) / (OD specificity control – OD control).

Measurement of human serum IgG fractions APA neutralization activity

Mouse monoclonal APA (mAPA510) 1 µg/ml was incubated with 100 µg/ml human IgG (isolated by Protein A sepharose from patient's serum) or with 100 µg/ml BSA as a control without inhibition. After 12 h of incubation we analyzed levels of free mAPA in aCL test using goat antimouse IgM peroxidase conjugate as a detector. Inhibition index was calculated for each sample = (OD mAPA with BSA / (OD mAPA with human IgG).

Measurement of *in vitro* serum APA neutralization by IvIg

Antiphospholipid antibodies positive serum samples 50 µl, taken before IvIg infusion, were incubated with 50 µl IvIg 1 mg/ml in PBS or 1 mg/ml BSA (control). After 12 h of incubation in 4°C samples were diluted 1 : 100 and tested for the APA as described previously. *In vitro* inhibi-

tion index was calculated for each sample = (OD 450 nm serum with BSA/OD 450 nm serum with IvIg).

Cytokine, immunoglobulins isotypes and circulating immune complexes determination

Tumor necrosis factor α, TNFR1, TNFR2, IL-6 and IL-4 were analyzed in serum samples taking before and after IvIg infusion by (BD Bioscience, San Diego, CA, USA) ELISA kits. Immunoglobulin G, IgM, IgA were determined in same serum samples by radial immunodiffusion, CIC levels were determined by (Quidel, San Diego, CA, USA) ELISA kit.

Results

Anti-idiotype Ab

Using anti-idiotype Ab binding ELISA we showed significant AiAPA presence in human serum in all examined groups. AiAPA bound to mAPA510 immobilized on plate and idiotype-antiidiotype reaction was specifically reduced by mAPA presence in sample diluents (specificity control) but was not sensitive to irrelevant monoclonal Abs (1C1) presence (Fig. 1). Most of the serum samples have 3-5 fold increased optical density in detection wells compared to negative and specificity control wells.

Results obtained in anti-idiotype-Ab-binding ELISA were confirmed in neutralization test. We showed that IgG fractions from 24 patients' serum inhibit reaction between mAPA and CL. Mean index of inhibition was 1.46 ±0.21 (data not shown). Anti-idiotypic antiphospholipid antibodies presence and neutralization activity was evident in samples from all patients' groups as well as in the healthy individuals. Analysis of the AiAPA levels depending on APA presence showed significant difference in groups. Mean AiAPA levels in APA positive IVF women and patients with CVHB were decreased as compared with APA negative patients from the same groups (Fig. 2). However no difference was founded in AiAPA levels in APA positive and APA negative SLE patients (Fig. 2). Anti-idiotypic antiphospholipid antibodies levels in healthy individuals were comparable with all patients' group.

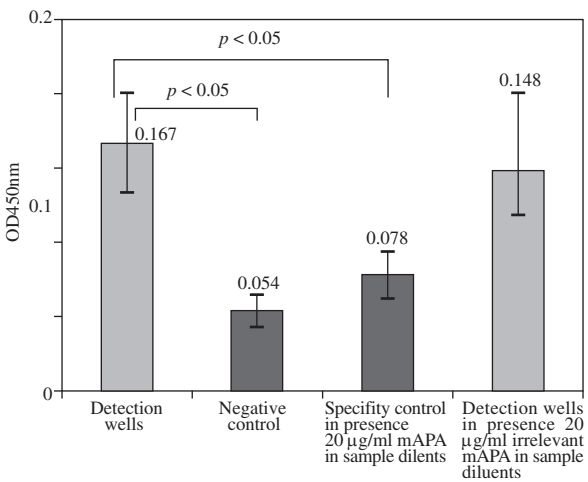

Fig. 1. Binding of AiAPA from patients serum (n = 235) to mouse monoclonal APA (mAPA) immobilized on plate (detection well) or to irrelevant mAb (negative control). Interaction significant reduced by presence 20 µg/ml mAPA in sample diluents (specificity control) but wasn't sensitive for irrelevant mAb presence

Intravenous immunoglobulin effect

We have shown significantly decreased APA and increased AiAPA levels in samples, that were taken 5 days after IvIg infusion, in comparison with preceding samples (Fig. 3). Intravenous immunoglobulin infusions induced noticeable APA decrease in 11 patients from 18. In 4 patients increased APA were observed and in 3 cases IvIg infusion had no effect on APA levels. In the same time increase of AiAPA in 9 and decrease in 2 from 11 patients was observed after IvIg infusion (Fig. 4). Antiphospholipid antibodies ratio (APA level after IvIg/APA level before IvIg) and AiAPA ratio (AiAPA after IvIg/AiAPA before IvIg) were calculated for each patient. We founded a significant negative correlation (r = –0.61) between APA and AiAPA ratios (Fig. 5).

The levels of Th1 cytokines (TNF, IL-6) and receptors TNFR1, TNFR2 did not change after IvIg infusion, only IL-4 significantly decreased after 5 days (1.7 ±0.4 pg/ml) as compared with previous level (3.2 ±0.6 pg/ml). No changes in serum levels of IgG, IgM, IgA and CIC were observed (data not shown). For investigation of IvIg treatment mechanism and causes of individual sensitivity to IvIg we elaborated the test for measuring of *in vitro* serum APA neutralization by IvIg. We showed that incubation of patients' serum with IvIg resulted to significant reduction

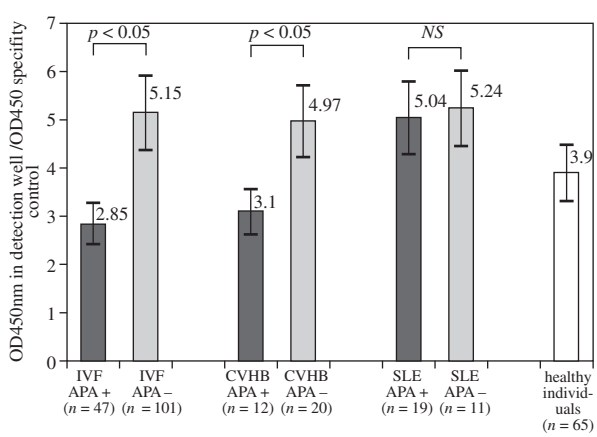

Fig. 2. AiAPA levels in APA (CL/PS) positive (APA+) or APA negative (APA–) patients groups

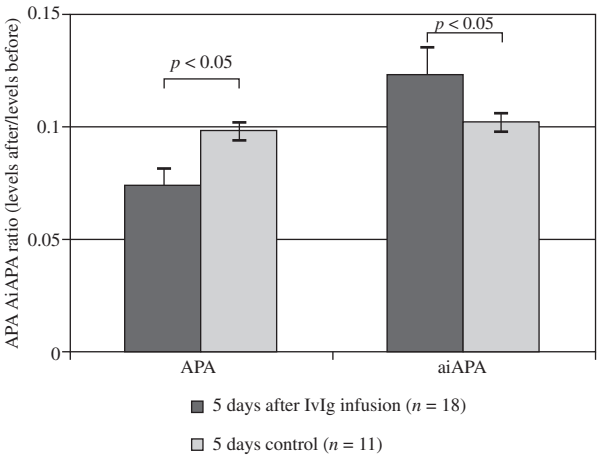

Fig. 3. APA ratio and AiAPA ratio 5 days after IvIg infusion and on 5 day interval in control APA positive patients without IvIg treatment
APA ratio = (aCL + aPS levels after IvIg infusion) / (aCL + aPS levels before IvIg infusion) AiAPA ratio = AiAPA levels after /AiAPA levels before IvIg infusion

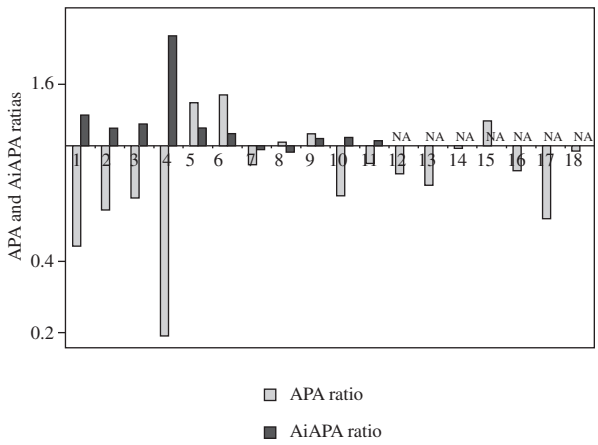

Fig. 4. Individually changing in APA and AiAPA ratios 5 days after IvIg infusion
APA ratio = (aCL + aPS level after IvIg infusion) / (aCL + aPS level before IvIg infusion) AiAPA ratio = AiAPA level after /AiAPA level before IvIg infusion

of APA levels (mean reduction = 34%, data not shown). *In vitro* reduction of APA levels in sera, which were taken before IvIg infusion, significantly correlated with APA decreased index after IvIg treatment (Fig. 5).

Antiidiotypic antibodies against natural cofactor-independent antiphospholipid antibodies are normally present in patients' serum (Figs. 1 and 2). These antibodies bind APA and neutralize their functional activity (Fig. 1) and possibly take part in regulation of APA production.

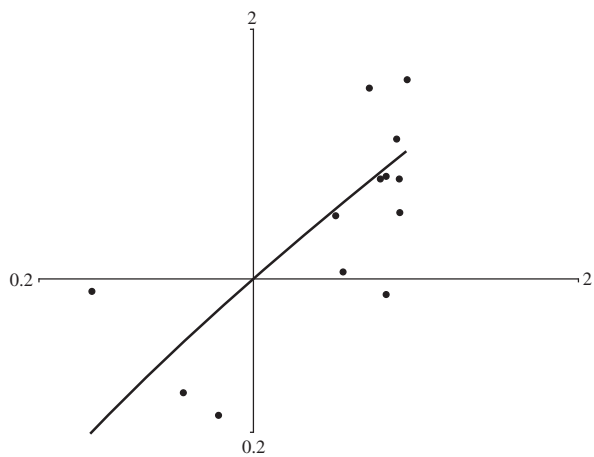

Fig. 5. Correlation of *in vitro* APA inhibition by IvIg preparation with APA decrease after IvIg infusion *in vivo*
APA ratio = (aCL + aPS level after IvIg infusion) / (aCL + aPS level before IvIg infusion)
Index of serum APA inhibition by IvIg *in vitro* = (OD serum + IvIg) / (OD serum + BSA)
Correlation $r = -0.61$; $p < 0.05$

Discussion

This study, performed by a specific technique, demonstrated the presence of AiAPA in human serum. We show AiAPA presence in serum and association between AiAPA levels and APA positivity. The analogous situation was described in idiopathic thrombocytopenic purpura patients where anti-idiotypic antibodies against anti-platelet antibodies led to clinic remission [29] and in patients with hemophilia A were anti-idiotypic antibodies took part in immune tolerance induction [30]. We also showed normally present AiAPA in healthy individuals.

It was showed earlier by Stea *et al*. [31, 32] that antiidiotypic antibodies which are present in healthy individuals may neutralize autoantibodies and prevent the emergence of pathogenic autoantibodies. Moreover, it was reported that antiidiotypic antibodies in maternal serum protect the fetus from anti-La/SSB antibodies by blocking pathogenic maternal autoantibodies [32].

Idiotypes and anti-idiotypic antibodies are natural components of immune responses, and exert a regulatory role which maintains the homeostasis of the immune system. These interactions between idiotypes and anti-idiotypic antibodies constitute an idiotypic network. Immunological balance might be found in the subtle equilibrium between APA and corresponding anti-idiotypic antibodies. Since normal individuals produce both APA and corresponding anti-idiotypic antibodies, the cause of the tolerance to phospholipids is not limited to deletion of self-reactive B and T cells. It might thus be fruit-

ful to investigate whether an idiotypic network plays a role in establishing and maintaining tolerance [30].

The results obtained in present investigation and data published earlier [33] suggest that AiAPA-APA system takes part in development and regulation of APA. Moreover, there are experimental data supporting the idea that APA and AiAPA are produced in physiological condition. Antiphospholipid antibodies negative sera obtained from normal donors can be converted to APA positive by heating the sera at 56°C for 30 min [34]. Normally present APA-AiAPA balance can be upset by infection, medication or, as in mice in experimental APS induction, by apoptotic cells infusion or corticosteroids treatment. This "transitory" APA [35] increased-balance ordinarily returned in normal APA-AiAPA ratio. But it is possible, that in some cases APA-AiAPA misbalance is starting conditions of true autoimmune state development.

Since binding of autoantibody to one component of a multicomponent complex can influence the subsequent processing and presentation of the other antigens in the complex [36], it is possible that coating of apoptotic blebs by aCL enhances the immunogenicity of these autoantigens [37]. When apoptosis occurs in a microenvironment in direct contact with the plasma, the procoagulant role of the apoptotic surface may be expressed additionally [37]. Opsonization of apoptotic cells by antiphospholipid antibodies has recently been shown to enhance recognition and phagocytosis by macrophages, with massive TNF-α secretion [36, 38]. The release of TNF-α may amplify this process by inducing further apoptosis and promoting the maturation of APC towards a more efficient antigen processing and presentation capability. The clearance of disrupted cell membranes from physiologically dead cells, such as PBL, may account for the exposure of CL and the subsequent production of autoantibodies [40].

In our opinion, regulation of APA by AiAPA is connected generally to cofactor-independent APA. Systemic lupus erythematosus patients with APA (generally β2GP1 dependent) have the same AiAPA level as APA negative ones. It is possible that cofactor-dependent APA in "true autoimmune response" form high idiotype heterogeneity and escape from anti-idiotypic control or this autoimmune response is supported by T-cell tolerance derangement.

It is rather possible that cofactor-independent APA are a part of normal physiological response against T-independent autoantigen like phospholipid-exposing cells in apoptotic or activation state. Plasmolemma with negatively charged phospholipid molecules on outer side form Ag structure that answers for all T independent Ag conditions. Classic inducers of experimental antiphospholipid syndrome in mice are apoptotic thymocytes [40] that represent on their surface negativly charged structure with polymeric repeating epitopes. This structure in association with all necessary co-stimulatory molecules may induce T-independent immune response. Possibly, B cells in this case haven't any variant except production of APA. This response is also downregulated in T-independent manner, by anti-idiotypic network. Currently, no anti-phospholipid-specific T cell have been described in contrast to cofactor-dependent APA production [41, 42].

Low doses IvIg treatment had normalizing effect on APA-AiAPA balance, not changing the levels of Th1 cytokines (TNF, IL-6), receptors TNFR1, TNFR2 and IgG, IgM, IgA, CIC, but gently decreased IL-4 levels. We showed that anti-idiotypic immunomodulation is the basic mechanism of (low dose) IvIg treatment in APA positive patients. Intravenous immunoglobulin infusion intensifies AiAPA function and results in to normalization AiAPA-APA balance. We did not find classical Th2/Th1 shift as described in cases of high dose treatment on mice model [22]. The levels of Th1 cytokines were not increased after IvIg infusion but IL-4 levels were gently decreased. IvIg deceased levels of APA *in vitro* and *in vivo*. *In vitro* effect of IvIg on serum can be used as a prognostic model for selection of IvIg treatment or dose definition. It also opens the way for more specific autoantibody treatment by anti-idiotypic Abs.

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