

The Prevalence and Clinical Significance of IgA Anti-Phosphatidylserine/Prothrombin Antibodies in Systemic Autoimmune Diseases

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Abstract

Objective: Studies on antiphospholipid antibodies have mainly focused on the IgG and IgM isotypes, with only a few investigating the pathogenic significance of IgA antiphospholipid antibodies. Positive IgA anticardiolipin (aCL) and IgA anti-β2 glycoprotein I (anti-β2GPI) were reported to be predominantly associated with other antiphospholipid antibodies, making it difficult to understand the role of IgA alone. Recently, antibodies against phosphatidylserine/prothrombin (aPS/PT) IgG and IgM have been indicated as a potential marker for antiphospholipid syndrome (APS). Our previous study reported that IgG and IgM aPS/PT showed highest association with lupus anticoagulant (LA) activity of all tested antiphospholipid antibodies, while no studies to date have investigated possible clinical benefits of IgA aPS/PT. In this study, we determined the prevalence of IgA aPS/PT in patients with systemic autoimmune diseases and evaluated their clinical association to thrombosis and obstetric complications.

Methods: 254 patients with systemic autoimmune diseases were screened for LA, aCL, anti-β2GPI and aPS/PT (for IgG, IgM, IgA isotypes).

Results: An overall prevalence of 63/254 (25%) was found for IgA aPS/PT in our cohort of patients. IgA aPS/PT were statistically significantly associated to LA activity and to both arterial and venous thrombosis, however no association was found to obstetric complications. Median levels of IgA aPS/PT were significantly higher in APS patients than in the non-APS patient control group comprising systemic lupus erythematosus, rheumatoid arthritis and Sjogren's syndrome patients.

Conclusion: Although IgA aPS/PT were predominantly associated with other antiphospholipid antibodies this study first confirmed their presence in APS patient samples and also showed a clear association of IgA aPS/PT to thrombosis and LA activity.

Keywords: Antiprothrombin antibodies; Phosphatidylserine-dependent antiprothrombin antibodies; Antiphospholipid antibodies; Enzyme immunoassays; Antiphospholipid syndrome

Introduction

Patients with antiphospholipid syndrome (APS) experience vascular thrombosis and/or pregnancy complications, associated with elevated levels of antiphospholipid antibodies [1]. These are a heterogeneous group of antibodies, comprising of IgG, IgM and IgA isotypes, which are directed against different plasma protein-phospholipid complexes or a single plasma protein. Current classification criteria for definite APS recommends the use of three laboratory assays, lupus anticoagulants (LA), IgG/IgM anticardiolipin (aCL) and IgG/IgM anti-β2-glycoprotein I antibodies (anti-β2GPI) to detect antiphospholipid antibodies in the presence of at least one clinical manifestation (i.e. venous, arterial, small vessel thrombosis or pregnancy complications, such as recurrent miscarriages, stillbirth or premature delivery due to preeclampsia or eclampsia). Antiphospholipid antibodies of the IgA

class are not recognized as formal laboratory criteria for APS, since in 2006 the consensus report concluded that available data is inadequate for establishing IgA as an independent risk factor for APS [1]. The following literature on IgA antiphospholipid antibodies is quite diverse, reporting a high variability in its prevalence, as well as clinical significance. However, several experimental data published recently showed possible pathogenic roles of both IgA aCL and IgA anti-β2GPI [2-7]. These antibodies have been reported in up to 70% of patients with systemic lupus erythematosus (SLE) and in those with primary APS [8]. The reports on the prevalence of IgA aCL are extremely variable ranging from 0% to nearly 50% [9]. Altogether, twelve studies showed an association between IgA aCL and certain clinical features related to APS, however fifteen studies failed to find any relationship between the presence of IgA aCL and clinical signs of APS (review in [9]). Studies investigating the diagnostic applicability and added value of IgA aCL show a general weakness, since elevated levels of IgA aCL are often accompanied with positive IgG and/or IgM aCL making it difficult to understand the single role of elevated IgA aCL. There is also added controversy in the literature regarding the

meaning of elevated IgA anti-β2GPI and recently Tebo et al. [10] also emphasized the variability in the performance characteristics of commercial kits for the detection of IgA anti-β2GPI. The majority of published papers have highlighted the value of IgA anti-β2GPI in the diagnosis of APS [9]. Thrombosis, particularly arterial thrombosis [6,11,12] is frequently found associated with IgA anti-β2GPI, although the simultaneous presences of other isotypes makes interpretation difficult. Mehrani et al. [13] reported on high prevalences of IgA antiphospholipid antibodies with 20% positive IgA anti-β2GPI associated with deep vein thrombosis. Lakos et al. [14] found that livedo reticularis, heart valve disease, thrombocytopenia and epilepsy are more common among subjects with increased IgA anti-β2GPI antibodies. The importance of IgA anti-β2GPI was also implicated for women undergoing in vitro fertilization treatment [7]. IgA anti-β2GPI seem to be more prevalent in SLE patients as compared to IgA aCL (review in [8]). Therefore, IgA anti-β2GPI have gained clinical relevance and were recently included among the antiphospholipid antibodies tests in the novel SLICC classification criteria for SLE [15]. For that reason, in selected patients where clinical suspicion of APS is high (but all other antiphospholipid antibodies tests are negative) testing for IgA anti-β2GPI might be clinically useful and therefore recommended [16-18].

Prothrombin is, in addition to β2GPI, an important antigen target for antiphospholipid antibodies. Although antiprothrombin antibodies have not yet been included in the classification criteria of APS, they are emerging as an increasingly important supportive marker [1,19-21]. We have previously reported of IgG and/or IgM antibodies against complex phosphatidylserine/prothrombin (aPS/PT) exhibiting the highest percentage of LA activity, as compared to aCL or anti-β2GPI [19] and their strong association to thrombosis and adverse pregnancy outcome, irrespective of other antiphospholipid antibodies [20,22]. The latest systematic review concluded that routine

measurement of aPS/PT antibodies of IgG and/or IgM isotype in serum or plasma might be useful in establishing the thrombotic risk of patients with previous thrombosis and/or (SLE) [21]. In addition a very recent international multicenter study advised measurement of IgG aPS/PT which might contribute to a better and more complete identification of patients with APS [23]. On the other hand no data on prevalence and clinical significance of IgA aPS/PT is available, therefore the present study aimed to investigate it in our population of patients with systemic autoimmune diseases.

Patients and Methods

Patients

We selected 131 consecutive APS patients classified according to the international classification criteria [1], 91 APS and 40 APS secondary to SLE seen in the Department of Rheumatology (University Medical Centre, Ljubljana) from 2006 to 2009. The control subjects were selected from consecutive non-APS patients who visited the same department from August to December 2009 and consisted of 47 SLE, 57 rheumatoid arthritis (RA) and 19 Sjögren's syndrome (SS) patients (Table 1).

Among all patients 55 subjects experienced arterial thrombosis, 60 venous thrombosis and 54 had a history of obstetric complications. Patients provided informed consent and had their sera collected when they were examined in the Department of Rheumatology (University Medical Centre, Ljubljana) and subsequently analyzed in the Immunology Laboratory. Sera from 221 apparently healthy blood donors were selected as the second control group. The study was approved by the National Medical Ethics Committee, Ljubljana, Slovenia (#99/04/15).

		Healthy controls n=221	APS n=91	APS+ SLE n=40	SLE n=47	RA n=57	SS n=19
Mean age + SD		42.8 ± 11.2	42.3 ± 15.3	44.4 ± 14.7	44.2 ± 15.2	57.9 ± 12.2	53.1 ± 18.5
Thrombosis		0	66	35	7	3	1
	Arterial Thrombosis	0	30	19	5	1	0
	Venous Thrombosis	0	37	18	2	2	1
Pregnancy loss defined by APS criteria [1]		0	37	13	3	0	2
	≥3 consecutive miscarriages <10th WG	0	8	1	0	0	0
	Fetal death >10th WG	0	14	2	0	0	0
	Premature birth <34th WG	0	6	0	2	0	0

Table 1: Patients' clinical features; APS: Anti Phospholipid Syndrome; SLE: Systemic Lupus Erythematosus; RA: Rheumatoid Arthritis; SS: Sjögren Syndrome; WG: Week of Gestation.

Measurement of antiphospholipid antibodies

All patients' and control sera were tested for IgG, IgM and IgA antiphospholipid antibodies in fresh sera either within 3 days of extraction or kept frozen at -20°C and immediately analyzed after first thaw.

In-house aPS/PT ELISA was performed as previously described [19]. Medium binding plates (Costar, Cambridge, USA) were coated with phosphatidylserine in chloroform/methanol 1:4 and dried overnight at 4°C . Following blocking with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 5 mM CaCl_2 (1% BSA/TBS-Ca), 25 μl of human prothrombin (Enzyme Research Laboratories, Ltd, Swansea, UK) (20 mg/L) and 25 μl of patients' sera diluted 1:50 were applied to wells immediately one after the other and incubated for 1 hour at room temperature (RT). After, alkaline phosphatase-conjugated goat anti-human IgG/IgM/IgA (ACSC, Westbury, USA) were applied in 1% BSA/TBS-Ca and incubated for 30 min. Detection of bound antibodies was made using paranitrophenylphosphate (Sigma Chemical Company, St. Louis, USA) in diethanolamine buffer (pH 9.8) and OD405 was kinetically measured by an ELISA reader (Tecan Sunrise Remote, Grödig, Austria).

In-house aCL ELISA was performed as previously described [24]. Briefly, medium binding polystyrene microtitre plates were coated with cardiolipin (CL) and blocked with 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). After washing with PBS, diluted samples in 10% FBS-PBS were applied and incubated at RT for 2.5 hours. The detection system was the same as in aPS/PT ELISA.

In-house anti- β 2GPI ELISA was performed as previously described [25] and evaluated through the European forum for antiphospholipid antibodies [26]. Briefly, high binding polystyrene microtitre plates coated with 50 μl /well of β 2GPI (10 mg/L) in PBS were incubated for two hours at RT. The plates were then washed with PBS containing 0.05% Tween-20 (PBS-Tween) and incubated with samples diluted in PBS-Tween for 30 min at RT. The detection system was the same as in aPS/PT ELISA.

Lupus anticoagulant

The assay was performed in blood samples collected in tubes containing 0.109 M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2400 g for 20 min at 4°C . After filtration, aliquots were stored at -80°C until use. Clotting tests were performed using coagulation analyzer BCS Siemens according to the previous guidelines of the International Society on Thrombosis and Haemostasis ISTH [26]. Simplified Dilute Russell's Viper Venom Test (dRVVT) was performed using LA1 Screening reagent and LA2 Confirmatory reagent (Siemens) following manufacturer's instructions [27]. A dRVVT ratio (LA1 screen/LA2 confirmation) above 1.2 was considered positive for LA activity. Activity of LA was quantified as follows: low positive (LA1/LA2=1.2-1.5), medium (LA1/LA2=1.5-2.0) and high positive (LA1/LA2>2.0).

Statistical analysis

Statistical analysis was performed using the SPSS 15.0 program. Normal distribution was evaluated using descriptive statistic parameters, curve fittings, and Kolmogorov-Smirnov test. The Receiver Operating Characteristic (ROC) analysis and the area under the curve (AUC) were used to assess the diagnostic performance of the measured marker(s). The results of multivariate logistic models were

approximated by odds ratio with its 95% confidence interval (OR) [95%]. A 2-sided P value <0.05 was considered statistically significant.

Results

The in-house IgA aPS/PT ELISA was validated according to recommended best practices for immunoassays for measurement of aCL and anti- β 2GPI published as a report from 13th International congress on Antiphospholipid Antibodies [17].

Frequency distribution of test results and cut-off levels for IgA aPS/PT ELISA

The arbitrary units of IgA aPS/PT (AUA) measured in 221 apparently healthy subjects were slightly bimodal and slightly right asymmetrical with outliers (Figure 1). The hypothesis of a normal distribution was rejected at 95% CI ($P<0.001$). Therefore, the cut-off levels for IgA aPS/PT were determined based on the 99th percentile of AU of main peaks. Samples with results above 4.5 AU were considered positive.

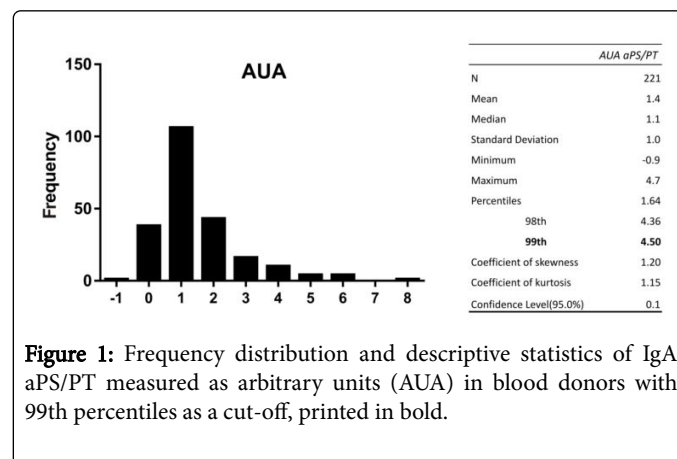


Figure 1: Frequency distribution and descriptive statistics of IgA aPS/PT measured as arbitrary units (AUA) in blood donors with 99th percentiles as a cut-off, printed in bold.

Precision of IgA aPS/PT ELISA

Repeatability (within-run precision) for IgA aPS/PT, as variability observed when as many factors as possible were held constant, was on average $<10\%$.

The reproducibility (between-run precision) where factors are varied and measurements are carried out over several days was on average $<15\%$ (Data not shown).

Intra-assay and inter-assay variability of IgA aPS/PT ELISA

Intra-assay variability was assessed by the coefficient of variation (CV) of all samples, internal standards and controls tested in duplicates. Inter-assay variability was estimated by the CV of internal standards and positive controls included in all 22 runs. The average intra-assay variability was 4.4% and the average inter-assay variability was 10.5% (Table 2).

Clinical significance of IgA aPS/PT compared to other antiphospholipid antibodies

In our cohort of 254 patient with systemic autoimmune diseases, 193 (76.9%) sera were positive for at least one measured antiphospholipid antibody.

	Intra-assay variability CV (%)	Inter-assay Variability CV (%)
No. of data	796	22
average	4.4	10.5
maximum	22.0	19.4
97.5 % Confidence upper level	4.7	13.2

Table 2: Intra-assay and inter-assay variability for IgA aPS/PT.

The highest prevalence was found for aCL (51.2%) followed by anti-β2GPI (42.9%), aPS/PT (39.8%) and LA (31.9%). Positive levels of

aPS/PT were determined in 101 patient sera. 58/254 (23%) patients had positive IgG aPS/PT, 64/254 (25%) positive IgM aPS/PT and 63/254 (25%) positive IgA aPS/PT.

Among IgA aPS/PT positive patients 43/63 (68.3%) were classified as having APS according to the international classification criteria. 2/63 (3.2%) had a history of arterial thrombosis and 1/63 (1.5%) with a history of arterial thrombosis and obstetric complications. The latter patient was positive also for IgG aPS/PT and IgA anti-β2GPI, in addition to IgA aPS/PT and consequently did not fulfill the laboratory criteria for APS. Among 17/63 (27%) IgA aPS/PT positive patients without any thrombotic or obstetric clinical manifestations, 10 had SLE, 4 RA and 3 SS and among them six were singularly positive, while 11 had multiple antiphospholipid antibodies positivity.

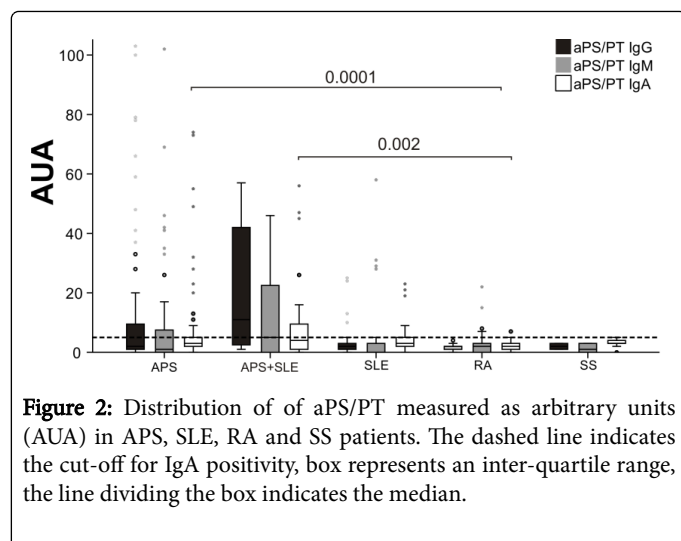
		Arterial thrombosis (n=55)		Venous thrombosis (n=60)		Obstetric complications (n=55)		Lupus anticoagulant activity	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
LA			ns	6.1 (3.0-12.2)	<0.001	3.7 (1.8-7.7)	0.001	/	/
aCL	IgG	3.5 (1.8-6.6)	<0.001	3.4 (1.8-6.3)	<0.001	4.1 (2.1-7.9)	<0.001	3.4 (1.9-6.0)	<0.001
	IgM		ns	2.2 (0.9-4.8)	0.048		ns	2.6 (1.2-5.7)	0.014
	IgA		ns	3.6 (1.8-7.3)	<0.001	2.5 (1.-5.6)	0.029	5.5 (2.5-12.4)	<0.001
anti-β2GPI	IgG	2.2 (1.2-4.1)	0.009	4.2 (2.2-7.7)	<0.001	2.7 (1.4-5.5)	0.002	5.1 (2.8-9.3)	<0.001
	IgM		ns		ns		ns	2.9 (1.2-6.2)	0.017
	IgA	2.0 (1.0-4.1)	0.046	3.2 (1.6-6.4)	0.001		ns	2.6 (1.3-5.0)	0.006
aPS/PT	IgG	2.2 (1.2-4.3)	0.016	4.9 (2.6-9.4)	<0.001	6.5 (3.0-14.0)	<0.001	18.4 (7.9-42.5)	<0.001
	IgM		ns	2.8 (1.5-5.3)	0.001	2.5 (1.2-5.1)	0.01	10.0 (5.0-20.3)	<0.001
	IgA	2.1 (1.1-3.9)	0.026	2.6 (1.4-4.9)	0.002		ns	4.7 (2.5-8.9)	<0.001

Table 3: Antiphospholipid antibodies in relationship to arterial thrombosis, venous thrombosis obstetric complications and lupus anticoagulant activity; aCL: Anticardiolipin antibody; Anti-β2GPI: antibodies against β2-glycoprotein I; aPS/PT: Anti-phosphatidylserine/prothrombin antibodies; CI: Confidence interval; ns: Not significant; OR: Odds ratio.

Median levels of IgA aPS/PT were 3.0 arbitrary units (AUA) in APS patients and 2.0 AUA in the non-APS patient control group comprising SLE, RA and SS patients; the distributions in the two groups differed significantly (Mann-Whitney U=6443, P=0.0071 two-tailed). Median levels of IgA aPS/PT did not differ between patients having APS only (primary APS) and patients having APS secondary to SLE. IgA aPS/PT was significantly higher in patients with primary APS, as compared to RA patients (Mann-Whitney U=1657, P=0.0001), as well as in patients with secondary APS compared to RA patients (Mann-Whitney U=725, P=0.0017) (Figure 2).

The positivity of individual antiphospholipid antibodies tests and specific clinical manifestations of APS were considered in the logistic regression analysis (Table 3). The correlation of individual antiphospholipid antibody and a specific clinical manifestation was calculated in the group of 254 autoimmune patients regardless of their

diagnosis. IgA aPS/PT were statistically significantly associated with arterial and venous thrombosis (OR 2.1, 95% CI (1.1-3.9), P=0.026 and OR 2.6, 95% CI (1.4-4.9) P=0.002, respectively). On the other hand, IgA aPS/PT were not found more prevalently in women with pregnancy complications (24.1%) than in those without (20.5%) (13/54 vs. 27/132, P=0.534). In patients with APS 43/131 (32.8%) had IgA aPS/PT, while 20/123 (16.3%) non-APS patients had IgA aPS/PT.



In this study there were 12 patients fulfilling clinical criteria for APS but lacking laboratory criteria for APS, specifically 7 SLE and 2 RA patients with history of thrombosis and 2 SLE and 1 SS with history of obstetric complications. Three of these 'seronegative' APS patients had IgA aPS/PT, two solely IgA aPS/PT and one SLE patient who exhibited a history of arterial thrombosis and obstetric manifestations, was also IgA anti- β 2GPI and IgG aPS/PT positive.

18/63 (29%) patients were solely positive for IgA aPS/PT and negative for IgM or IgG aPS/PT. Five of these patients (3 SLE, 1 RA and 1 SS) were concomitantly negative also for LA, aCL and anti- β 2GPI. Of these patients, one experienced arterial thrombosis. On the other hand, none of the tested patients was solely IgA aCL positive and five were solely IgA anti- β 2GPI positive, among which two experienced thrombosis (i.e. one arterial and one venous thrombosis). In patients with APS, the average number of positive antiphospholipid antibodies tested (LA, aCL, anti- β 2GPI and aPS/PT) was 2.4, while in the APS negative groups, this number was 0.85, implying that the diagnostic precision of APS is proportional to the number of antiphospholipid antibodies tests performed.

Discussion

In the present study, we show that IgA aPS/PT are present in 43/131 (32.8%) APS patients. IgA aPS/PT are statistically significantly associated to both arterial and venous thrombosis. No studies to date have investigated the prevalence and clinical significance of IgA antiprothrombin antibodies, but several studies have confirmed an association between IgA anti- β 2GPI and an increased risk of thromboembolic vascular disease [2-5,27]. In agreement with the last international consensus [1] we also demonstrated association of IgA anti- β 2GPI with thrombotic manifestations.

In the last decade, antibodies against prothrombin, specifically aPS/PT were recognized to be importantly associated with clinical manifestations of APS [20-23,28]. In our previous study we have reported that aPS/PT of IgG and IgM isotype are strongly associated with adverse pregnancy outcome, irrespective of other antiphospholipid antibodies [22], and that their detection is particularly important for evaluation of obstetric APS. However in the present study, we show that IgA aPS/PT (differently from IgG and

IgM) were not found more prevalently in women with pregnancy complications.

Our study also comprised of some patients with clear clinical features of APS but lacking persistently elevated criteria of antiphospholipid antibodies. These so called 'seronegative' APS patients possibly exhibit certain antiphospholipid antibodies unidentifiable with the current assays. Even if such a patient does not fully satisfy the classification criteria, he/she may still have APS. Missing a diagnosis of APS in these individuals may lead to the absence of appropriate therapy and potentially, significant adverse outcomes. Given the limitations of currently available antiphospholipid antibodies assays, more and more studies are investigating novel serological markers, such as antiprothrombin antibodies, for optimization of evaluation of potential APS patients. We found three 'seronegative' APS patients having IgA aPS/PT, among which two had only these antibodies elevated.

In 2006, an international classification criteria committee recommended IgG and IgM antiphospholipid antibodies, as laboratory criteria for APS [1]. A later systematic review of clinical relevance of IgA aCL and IgA anti- β 2GPI antibodies, published, by Meijida et al., concluded that according to data published at the time, there was not enough evidence recommended for routine testing these antibodies which would increase the diagnostic accuracy of the APS [5]. Despite current laboratory classification criteria for APS [1], clinicians will occasionally encounter patients with solely present IgA antiphospholipid antibodies who exhibit clinical manifestations of APS. In our study, IgA aCL, IgA anti- β 2GPI and IgA aPS/PT highly correlated to LA activity and were significantly associated to thrombosis. However, since our cohort of patients was selected according to pre-determined diagnosis, it did not allow us to reveal more than a few solely positive IgA patients. Since this is the first time IgA aPS/PT have been measured and reported, the limitation is the lack of methodology validation in different laboratories using larger cohorts of "seronegative" patients. So, further studies are needed to evaluate IgA aPS/PT levels systematically in the future. Although our study could not advise testing IgA aPS/PT to increase the diagnostic accuracy of APS, we clearly show that IgA aPS/PT are present in APS patients and are associated with an increased risk for thrombotic vascular events.

Conclusion

To summarize, based on our results IgA aPS/PT, IgA anti- β 2GPI and IgA aCL presented an independent risk factor for thrombosis and highly correlated to LA activity. Prospective studies measuring IgA antiphospholipid antibodies in patients with clinical manifestations of APS, negative for conventional antiphospholipid antibodies could better estimate their clinical value.

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