Case Report

Obstetric Antiphospholipid Syndrome: An Approach from Glycans of the Immunoglobulin G

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Abstract

This is a case report of women with pregnancy morbidity (PM), some of them associated with antiphospholipid syndrome (APS), in which the glycan patterns of immunoglobulin G (IgG) were investigated based on the theory of alteration of glycosylation in autoimmunity. We used lectin blot to determine changes in terminal glycosylation of polyclonal IgG from women with antiphospholipid (aPL) antibodies and PM plus vascular thrombosis (PM/VT) and seronegative-obstetric APS (SN-OAPS). In addition, we analyzed IgG from women with PM without aPL (PM/aPL-) and healthy women, as controls. Even though the SN-OAPS and PM/VT groups share the PM, only the SN-OAPS group showed a decreased expression of galactose compared to the healthy group. We also found the presence of mannosylated oligosaccharides in IgG from all patients being significantly higher in IgG from women of the PM/aPL$^-$ group. The differences in glycans presented here could relate to pathological mechanisms of PM associated with APS.

Keywords: *Antiphospholipid antibodies, antiphospholipid syndrome, glycosylation, immunoglobulins, pregnancy morbidity, thrombosis*

Introduction

Pregnancy morbidity (PM) and vascular thrombosis (VT) are clinical manifestations of the antiphospholipid syndrome (APS), an autoimmune disease characterized by the persistent presence of antiphospholipid (aPL) antibodies.[1] Immunoglobulin G (IgG) is the most common Ig isotype in aPL-positive women who attended the Recurrent Pregnancy Loss Program in our institution. IgG has structural differences and also a specific biological function, and these differences are influenced by both composition and linkage of the sugar residues. Altered glycan theory of autoimmunity states that each autoimmune disease will have a unique glycan signature present in the glycosylation patterns of the diverse Ig.[2] Changes are described for several autoimmune diseases,[3] and we hypothesize that seronegative-obstetric APS (SN-OAPS) has its own glycan signature in comparison with healthy women, and this could be associated with pathological mechanisms of the disease.

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Serum samples were obtained from women who attended the Recurrent Pregnancy Loss Program in our institution (n = 32). Our Ethics Review Committee approved the collection of the sera, and written consent was obtained from all participants. The clinical and laboratory features of women included in this study are outlined in Table 1. Women in this study were classified as Pregnancy Morbidity/Vascular Thrombosis (PM/VT), women that fulfilled the clinical and laboratory revised Sapporo classification criteria for APS (n=7); Seronegative-Obstetric APS (SN-OAPS),

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Table 1: Clinical and laboratory features of the women included

Parameter	NHS (*n*=7)	PM without aPL, PM/aPL- (*n*=10)	Seronegative-obstetric APS, SN-OAPS (*n*=8)	PM/VT (*n*=7)
Age (mean of years±SEM)	36.1±2.44	30.8±1.82	33±1.8	37.1±2,2
Previous fetal losses (mean and range)				
≤10 weeks of gestation	0	2, 1 (1–5)	1, 0 (1–4)	2, 0 (2–5)
>10 weeks of gestation	0	0, 4 (0–3)	0, 75 (1–2)	1, 4 (1–5)
Preeclampsia<34 weeks (*n*)	0	0	0	4
Arterial/venous thrombosis (*n*)	0	0	0	7
Lupus anticoagulant (mean±SEM)[£]	1.06±0.05	1.13±0.01	1.01±0.05	2.18±0.89
IgG aβ2GPI in a pool of serum (mean, SGU)[†]	3.69	4.36	4.71	85.76
IgG anti-cardiolipin in a pool of serum (mean, GPLU)[‡]	10.09	6.79	8.95	93.24
aβ2GPI in 100 µg/mL of IgG purified from sera (mean, SGU)[†]	2.84	3.1	4.15	14.64
Anti-cardiolipin in 100 µg/mL of IgG purified from sera (mean, GPLU)[‡]	6.43	2.98	7.77	16.98
Serum other no-criteria IgG antiphospholipid antibodies (mean, percentage of positive control)				
aPI	0	0	39.17	58.77
aPS	6.57	0	45.63	80.70
aPG	15.03	0	32.13	74.37
aPA	4.23	0	15.03	97.03

[£]Positive>1.2; [†]Positive at >20 SGU; [‡]Positive at >10 GPL; [§]Positive at>25%; *GPLU=Immunoglobulin G phospholipid units, SGU=Standard immunoglobulin G units, aPI=Anti-phosphatidyl inositol antibodies, aPS=Anti-phosphatidyl serine antibodies, aPG=Anti-phosphatidyl glycerol antibodies, aPA=Anti-phosphatidic acid antibodies, NHS=Normal human serum, PM=Pregnancy morbidity, APS=Antiphospholipid syndrome, SN-OAPS=Seronegative-obstetric antiphospholipid syndrome, VT=Vascular thrombosis, SEM=Standard error of mean, IgG=Immunoglobulin G, aβ2GPI=Anti-β2glicoprotein-I

women who present clinical features consistent with a diagnosis of obstetric APS but tested persistently negative for conventional anti-cardiolipin (aCL), anti-β2glicoprotein-I (aβ2GPI) and lupus anticoagulant (LA) tests[4], these women were positive for the non-criteria aPL[5] (n=8), and Pregnancy Morbidity/aPL- (PM/aPL-), women with a history of gestational morbidity, without autoimmune or chronic diseases, negative for both non-criteria aPL and conventional tests (n=10). These women were compared with a Normal Human Serum (NHS) group, healthy women with previous uncomplicated pregnancies (n= 7). All women were tested for aPL twice, at least 12 weeks apart.

Serum samples were pooled within the study groups, and polyclonal IgG was purified by affinity chromatography, as described elsewhere.[6] To confirm IgG purification, both capillary and conventional electrophoreses were performed. A 150 kDa peak was observed for each IgG in capillary electrophoresis test. In addition, conventional electrophoresis performed in reduced conditions allowed to observe two bands of 50 and 25 kDa that correspond to the heavy and light chains of IgG, respectively [Supplementary Figure 1].

To determine the glycan profile of purified IgG, a lectin affinity blotting analysis was performed using a DIG Glycan Differentiation kit (Roche, Basel, Switzerland) to detect and to characterize carbohydrate glycotope on proteins, according with an established protocol in our laboratory.[7] After gel electrophoresis, the proteins were transferred to a nitrocellulose membrane using a semi-dry blotter with Tris-glycine 1x (pH = 8.3) and 20% methanol at 116 mA per membrane. The transferred membranes were blocked with blocking solution 1x for 45 min and then incubated with each lectin coupled to digoxigenin for 1 h. *Galanthus nivalis agglutinin* (GNA), *Sambucus nigra agglutinin* (SNA), and *Datura stramonium agglutinin* (DSA) were diluted to 0.1% v/v in buffer 1 (Tris-buffered saline; 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ at pH 7.5); *Maackia amurensis agglutinin* (MAA) and *Peanut agglutinin* (PNA) were diluted to 0.5% and 1.0% v/v, respectively. Membranes were washed and then incubated with polyclonal sheep anti-digoxigenin secondary antibody conjugated to alkaline phosphatase 0.1% for 1 h. Finally, to detect the lectin–carbohydrate complex, the membranes were stained with 2% nitroblue tetrazolium solution supplied in the kit. The reaction was stopped with sterile water, and a dark precipitate was formed in the membrane. In order to perform a semi-quantitative analysis, the intensity of the bands was quantified by densitometry, which

indicates the amount of the terminal saccharide of the glycan of each IgG pool. ImageJ software (National Institutes of Health, Bethesda, USA) was used to perform densitometry analysis in the membranes. Then, we compared the mean intensity of these bands between the groups of study.

All experiments were performed at least three times. Data are expressed as the mean ± standard error of the mean. Statistical significance was determined with one-way ANOVA and Dunn's correction for multiple comparisons using GraphPad Prism 6.0 software (Software Inc., La Jolla, USA).

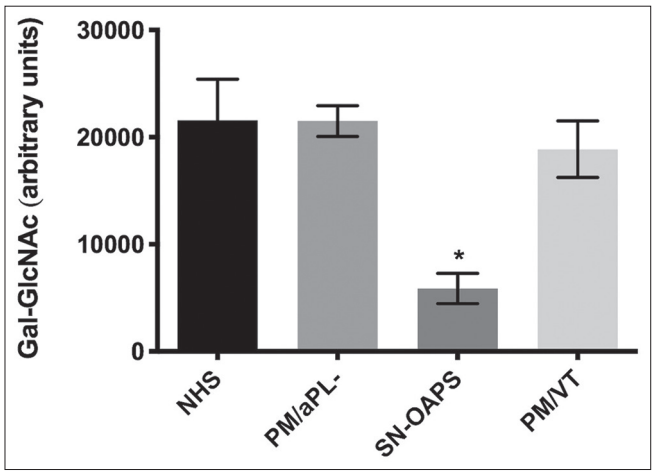

Figure 1: Expression pattern of terminal galactose

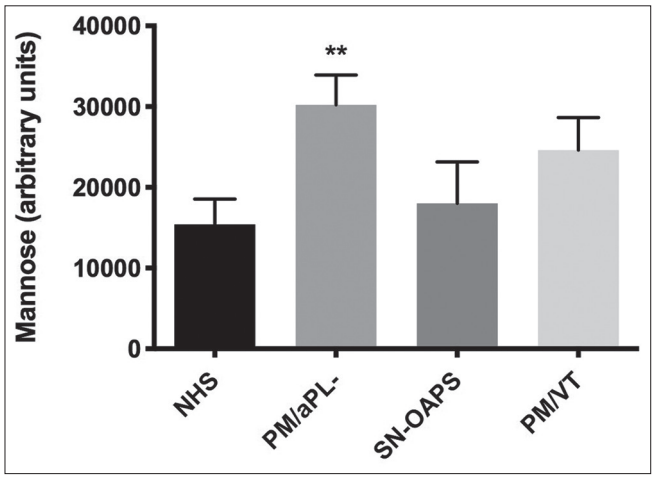

Figure 2: Expression pattern of terminal mannose

The amount of galactose β1-4 N-acetylglucosamine (Gal-GlcNAc) – detected by DSA lectin – was decreased in IgG from women SN-OAPS compared with healthy women ($P < 0.05$) [Figure 1]. This could be caused by an absence of the terminal galactose, indicating the presence of monogalactosylated and agalactosylated oligosaccharides. High levels of these oligosaccharides have been associated with pro-inflammatory features,[8] and the inflammatory state is well described in women with PM and aPL. Therefore, the obstetric features of APS could be related to this specific glycan profile in a kind of aPL in this case nonconventional aPL. This is an interesting finding, since we suspected different IgG populations inside the SN-OAPS and PM/VT study groups. In addition, we observed the presence of mannosylated oligosaccharides in IgG from all women who could be hybrids or highly mannosylated according to other studies. This amount of terminal mannose detected by GNA lectin was significantly higher ($P < 0.01$) in IgG from women from the PM/aPL$^-$group [Figure 2]. The increased presence of mannose residues could include distinct isomers of $Man_8GlcNAc_2$ (M8A, M8B, and M8C) and $Man_5GlcNAc_2$ (M5). IgG with high quantities of M5 and M8 has been involved in enhanced antibody-dependent cell-mediated cytotoxicity due to increased binding to the FcγRIIIA receptor in effector cells.[9]

Expression of sialic acid α2,6 was detected by SNA lectin. No statistical difference was found in this expression between the groups [Figure 3a]. SNA lectin allowed detecting glycosylation of both IgG heavy and light chains in all the groups, and interestingly, the light chain was not detected with any other lectin [Figure 3b]. The expression of sialic acid α2,3 – detected by MAA lectin – was evaluated, but

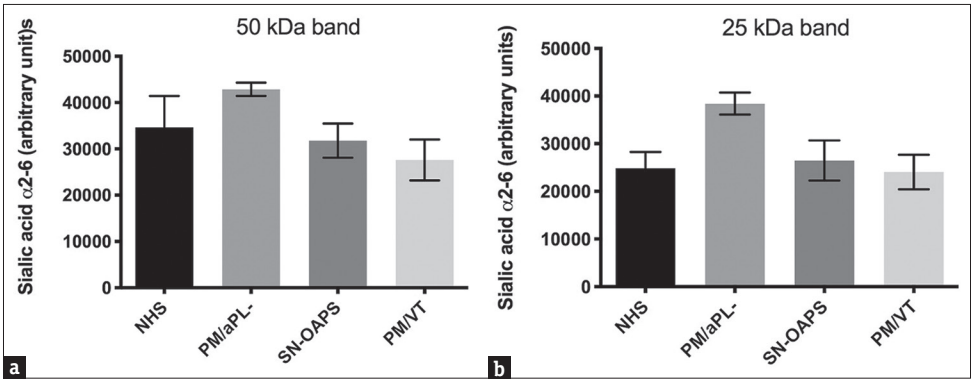

Figure 3: Expression of sialic acid α2-6 in both (a) heavy and (b) light chains of immunoglobulin G

no reactivity was observed indicating the absence of monosaccharide (data not shown). Finally, to determine the core 1 O-glycans, the amount of galactose 1-3 N-acetylgalactosamine detected by PNA lectin was evaluated, but as expected, no reactivity was found (data not shown).

Discussion

The search for biomarkers to APS diagnosis is expanding, but to date, clinical applications of proteomics have been focused on cardiovascular risk and thrombosis associated with APS rather than to OAPS.[10] Advances in proteomic technology could help to identify potential biomarkers of OAPS, and the study of glycobiology offers a complementary knowledge of protein function. Therefore, the search for glycan patterns of proteins involved in immune responses could be a novel pathway to understand the disease. We found some changes in glycosylation profiles in IgG from women with PM, specifically a reduction in terminal galactose that could be useful in the future. Furthermore, since we were considering a differentiated pathogenicity of the IgG in each study group, our results support that glycosylation differences in this IgG could alter their function and allow the trigger of several physiological pathways, leading to different effects on the cells and inducing a specific clinical manifestation.

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Conflicts of interest

There are no conflicts of interest.

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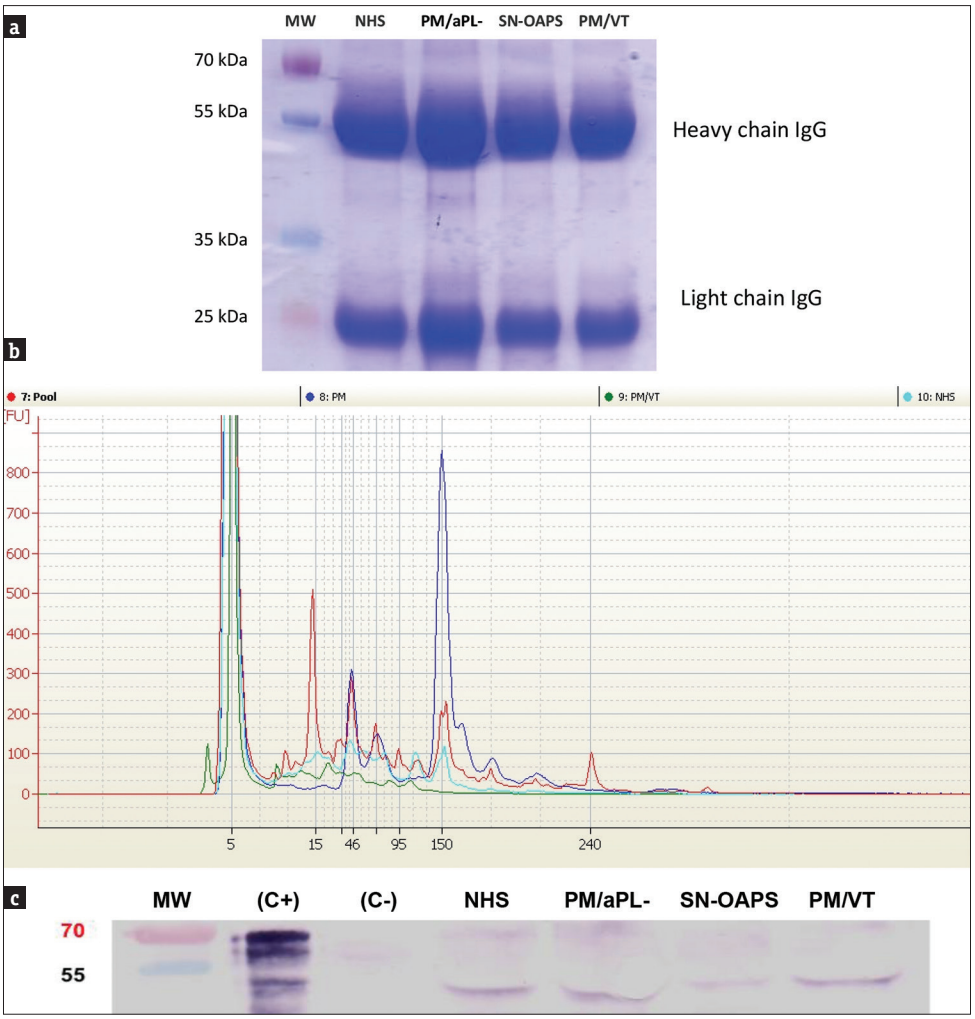

Supplementary Figure 1: (a)Representative image of conventional electrophoresis of immune-purified IgG. (b) Electropherogram of IgG samples from high sensitivity protein electrophoresis obtained with the 2100 Bioanalyzer system. Samples: Pool (PM/aPL-, red), PM (SN-OAPS, blue), PM/VT (green) and NHS (cyan). (c) Representative image of Lectin Blot using DSA lectin to detect Gal-GlcNAc. Fetuin and carboxypeptidase Y were used as positive control (C+) and negative control (C-) respectively