The efficacy of specific IVIG anti-idiotypic antibodies in antiphospholipid syndrome (APS): trophoblast invasiveness and APS animal model

Miri Blank1,2, Liat Anafi1, Gisele Zandman-Goddard3, Ilan Krause1, Shlomit Goldman4, Eliezer Shalev4, Ricard Cervera5, Josep Font5, Mati Fridkin6, Hans-Jurgen Thiesen7 and Yehuda Shoenfeld1,3,8

1The Autoimmune Disease Center, Sheba Medical Center and 2Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Israel
3Department of Medicine B, Sheba Medical Center, Tel-Hashomer and Sackler faculty of Medicine, Tel-Aviv university, Israel
4Laboratory for Research in Reproductive Sciences, Department of Obstetrics and Gynecology, Ha’Emek Medical Center, Afula, Israel
5Department of Autoimmune Diseases, Institute Clinic de Medicina i Dermatologia, Hospital Clinic, Barcelona, Catalonia, Spain
6Department of Organic Chemistry, The Weizman Institute for Sciences, Rehovot, Israel
7Department of Immunology, University of Rostock, Schillingallee 70, 18055 Rostock, Germany
8Incumbent of the Laura Schwarz-Kipp Chair for Research of Autoimmune Diseases, Tel-Aviv University, Israel

Keywords: antiphospholipid syndrome, autoantibodies, beta-2-glycoprotein-I, experimental model, IVIG

Abstract

Objectives: Administration of intravenous Ig (IVIG) is a recognized, safe and efficient mode of immunomodulatory therapy for many autoimmune diseases. Anti-idiotypic antibody binding to pathogenic autoantibodies and hence inhibition of binding to the corresponding antigen is one postulated mechanism of the beneficial effect of IVIG. The aim of this study was to fractionate the anti-beta-2-glycoprotein-I (β2GPI) anti-idiotypic antibodies from a commercial IVIG preparation and use it as a treatment in an experimental antiphospholipid syndrome (APS) mouse model. Methods: Anti-β2GPI polyclonal antibodies were purified on a β2GPI column. The purified antibodies were bound to CN–Br-activated sepharose and employed for purification of IVIG-anti-anti-β2GPI (anti-idiotypic antibodies), defined as specific intravenous Ig (sIVIG). The idiotype specificities were confirmed by competition assays. The effect of sIVIG in vitro was tested in a trophoblast and choriocarcinoma matrigel/invasion assay (i.e. proliferation and metalloproteinase (MMP)2/MMP9 expression) and in vivo in a fetal loss model of APS. Results: Anti-β2GPI antibodies inhibited human trophoblast cell invasion in vitro. The function was attributed to the Fab portion of the anti-β2GPI Igs, since β2GPI-related synthetic peptides specific for the Fab part of the anti-β2GPI antibodies neutralized its activity. APS sIVIG fraction reduce human trophoblast invasion in vitro by 560 times more than the whole IVIG compound and improved the MMP2 and MMP9 production by trophoblast cells. sIVIG improved significantly (200 times more) the pregnancy outcome in BALB/c mice passively infused with anti-β2GPI antibodies, in comparison to treatment with IVIG (P < 0.02). Conclusions: Based on the current results, we propose that APS sIVIG may be considered as potential specific therapeutic safe compound for developing a treatment for APS patient’s early fetal loss.

Introduction

The classical antiphospholipid syndrome (APS), ‘Hughes syndrome’, is characterized by the presence of anti-phospholipid antibodies which bind negatively charged phospholipids, platelets, endothelial cells and neuronal cells via beta-2-glycoprotein-I (β2GPI), associated with recurrent fetal loss, thromboembolic phenomena and thrombocytopenia (1–4). Recurrent fetal loss is one of the ‘Sapporo’ criteria for APS, encompass unexplained fetal death >10 weeks of pregnancy or three or more unexplained fetal loss prior to 10 weeks (5, 6). Currently, it is well established that anticardiolipin-β2GPI-dependent antibodies are the main cause for APS-related fetal loss and thrombosis (7–11). Passive
transfer of these antibodies into naive mice resulted in elevated percentage of fetal loss, impaired embryonic implantation and induction of other findings characteristic of APS (10–14).

For more than three decades, intravenous Ig (IVIG) preparations were reported to be beneficial in patients with a variety of autoimmune disorders (15–21), including a review on IVIG treatment in APS patients (16–18). The main mechanisms known so far to explain its broad activity comprise: (i) provision of anti-idiotypic antibodies and their function as immunomodulators; (ii) interference with the activation of complement and the cytokine network; (iii) modulation of the expression and function of Fc receptors and (iv) differentiation and effector functions of T and B cells, all summarized in (15, 18). Currently, it is well established that commercial IVIG preparations contain anti-idiotypic antibodies against a variety of idiotypes such as anti-factor VIII, anti-DNA, anti-intrinsic factor, anti-thyroglobulin, anti-neutrophil cytoplasmic antibodies, anti-microsomal, anti-neuroblastoma, anti-phospholipid, anti-platelet, anti-Sm idiotype (4B4), anti-GM1 and anti-desmoglein-3 antibodies (15–23). In the past specific intravenous Ig (sIVIG) preparations, prepared for anti-Fas, were already studied (24, 25). Previously, we have fractionated IVIG specific for anti-DNA anti-idiotypic antibodies, employing a column composed of anti-dsDNA affinity purified from 55 lupus patients at active stage of the disease. This IVIG fraction showed specific activity for systemic lupus erythematosus patient’s idiotypes in vitro and was 200 times more effective than the whole IVIG commercial compound in NZBxWF1 mice (26).

In this study, we attempted to fractionate IVIG-specific anti-idiotypic antibodies (anti-anti-β2GPI) from IVIG preparation (named sIVIG) and to study its biological functions in vitro and in an APS mouse model.

Methods

Cell cultures

JAR (HTB 144, American Type Tissue Collection) human choriocarcinoma cell line was established from trophoblast tumor of the placenta. Cells were grown in dodecyl maltoside-199 medium supplemented with 5% FCS and penicillin/streptomycin (Beit-Ha’Emek, Israel).

Human trophoblast cells were obtained from legal abortions (9–12 weeks of gestational age), with the approval of the local ethical committee (in compliance with the Helsinki Declaration) and the consent of the participating patients. Trophoblast cells were isolated as described previously in detail elsewhere (27, 28).

Synthetic peptides

The following β2GPI-related peptides were used in the study: Peptide A: LKTRPV65(12), B: DKATP121(11), C: TLRVYK13 (12), D: GDKVSCFCKNKEKKC128 (29) and E: KEHSSLAFWK173 (30). The D form of the synthetic peptides was used as control peptide. The concentration of each peptide in the used cocktail was 10 μg. The peptides were prepared by conventional solid-phase peptide synthesis, using an ABIMED AMS-422 automated solid-phase multiple peptide synthesizer (Langenfeld, Germany). For purity determination, analytical reversed-phase HPLC was performed using a prepacked-100 RP-18 column (Merck, Darmstadt, Germany) (12).

Igs

IVIG—IVIG preparation named OMRIGAM was kindly provided by Omrix Pharmaceutical Ltd, Nes-Ziona, Israel. C-IgG—IgG from one healthy individual was loaded on anti-human-IgG sepharose column (Sigma), the bound IgG was eluted by using 5 M MgCl2 and dialyzed against PBS pH 7.4.

The sIVIG, IVIG, C-IgG or anti-β2GPI antibodies were biotinylated as described before (12).

F(ab)2 fragments

IVIG was dialyzed against 100 mM Na-acetate buffer, pH 4.0 and digested with pepsin (2% w/w, Sigma chemical Co., St Louis MO, USA), at 37°C for 18 h. Any remaining traces of undigested IgG and Fc fragments were removed by binding to a protein-A column (Pharmacia Biotech, Norden AB Sollentuna, Sweden). The efficiency of the IVIG digestion was confirmed by running on 10% SDS-PAGE.

Fractionation of sIVIG from IVIG preparation

APS sIVIG, anti-anti-β2GPI fraction of IVIG was prepared in three stages.

Stage I. Preparation of β2GPI. Plasma from a healthy donor were incubated with 60% perchloric acid for protein sedimentation, neutralized with NaHCO3 and dialyzed against PBS. The dialyzed proteins were loaded on a Heparin column. The β2GPI was eluted with Tris buffers pH 8 0.15 M, 0.35 M and 0.50 M. The fractions were further purified on protein-G column (Pharmacia). The protein concentration was defined by BCR™ protein assay kit (Pierce, Rockford, IL, USA) and tested for purity by western and immunoblot analyses. The purified β2GPI was used for construction of β2GPI column using activated CN-Br-activated Sepharose 4B (Pharmacia) according to the manufacturer’s instructions.

Affinity purification of anti-β2GPI antibodies. Human anti-β2GPI antibodies were affinity purified from 15 patients with APS at an active stage of disease (the blood was taken when the patients experienced episodes of pregnancy loss and/or thrombosis). The plasma was passed on β2GPI column, elution with Glycin–HCl pH 2.5, neutralization with Tris and dialysis against PBS. The binding of anti-β2GPI to β2GPI was confirmed by ELISA and inhibition studies.

Stage II. Construction of anti-β2GPI column. A mixture of affinity-purified anti-β2GPI antibodies from 15 patients with APS were used since different APS patients’ sera entail diverse anti-β2GPI anti-idiotypic antibodies. The mixture of anti-β2GPI antibodies was dialyzed against coupling buffer (0.1 M NaHCO3 pH 8.3 containing 0.5 M NaCl) overnight at 4°C and covalently bound to CN-Br-activated Sepharose 4B. The remaining active NH groups were blocked by incubation with 0.2 M Glycine, pH 8.0 overnight at 4°C. Three cycles of washings with coupling buffer followed by 0.1 M acetate buffer pH 4.0 was used to remove the excess of
unbound Ig. The washed column was equilibrated in Tris buffer pH 7.4.

Stage III. Fractionation of IVIG anti-anti-β2GPI anti-idiotypic antibodies—sIVIG. Five hundreds milligrams of the commercial IVIG preparation (Omrix, Nes-Ziona, Israel) were dia-lyzed against loading buffer (0.05 M Tris containing 0.5 M NaCl, pH 8.0), diluted in 50 ml of loading buffer, filtered through the 0.45-µm filter (Minisart, Sartorius AG, Germany) and passed through the anti-β2GPI column for 16 h at 4°C. Unbound material was washed out and the bound antibodies were eluted with 0.2 M Glycine–HCl, pH 2.5 and neutralized with 2 M Tris. The IgG containing fractions were pooled, dialyzed against PBS and tested for anti-idiotypic activity.

The anti-idiotypic binding sIVIG was analyzed by direct binding of biotinylated sIVIG or commercial IVIG to anti-β2GPI bound to goat-anti-human-Fc-coated ELISA plates. The inhibition of anti-β2GPI binding by sIVIG in comparison to IVIG was analyzed by inhibition assays. sIVIG, IVIG and IgG affinity purified from a single donor (C-IgG) were used as competitors and tested for their ability to inhibit the binding of biotinylated human anti-β2GPI affinity purified from five patients to β2GPI-coated ELISA plates.

Matrigel invasion assay

Matrigel invasion assay was performed as previously described in the literature (27, 28), briefly: Matrigel (100 mg ml⁻¹) (BD Biosciences, Beit-Ha’Emek, Israel) in serum-free cell culture media was added to upper chamber of 24-well transwell plate (Corning) and incubated at 37°C 3–4 h for gelling. JAR cells were harvested from tissue culture flasks by Trypsin/EDTA, washed and resuspended in 5% FCS in M-199 medium and added to upper wells at a amount of 10⁵ cells per well in 200 μl medium, while 500 μl medium was added to lower well. First trimester trophoblasts were cultured in upper wells at a density of 2 × 10⁵ cells per well in 100 μl medium. The same density of cells, in the absence or presence of anti-β2GPI 10 mg ml⁻¹, was seeded in a well without transwell and counted at time of the invasion assay, as reference of total cells. IVIG, sIVIG or C-IgG at different concentrations were added to medium in upper and lower wells. Plates were incubated at 37°C for 48 h for JAR cells and 72 h for trophoblasts. Then, the non-invaded cells on top of the transwell were scraped off with a cotton swab. The amount of invaded cells in the lower wells as a percent of total seeded cells was evaluated with XTT Reagent kit (equivalent to MTT kit). The percent of invasion was cal-culated as: [absorbance of invaded cells/absorbance of seeded cells] ×100 = invasion (%). Inhibition of invasion was calculated as percent of invasion in the presence of Ig from percent of control invasion without Ig.

Proliferation assay

Evaluation of cell proliferation was performed with XTT Reagent kit (XTT, cell proliferation kit, Beit-Ha’Emek, Israel, equivalent to MTT kit) according to the manufacturer’s protocol.

Specific IVIG in experimental antiphospholipid syndrome

Zymography (substrate-gel electrophoresis)

In order to detect proteolytic activity of matrix metalloprotei-

nases (MMP2, MMP9) in conditioned media (CM) collected after 48–72 h culture, substrate-gel electrophoresis (Zymography) on gels containing gelatin as the substrate were used as was previously described (31): CM were electrophoresed through a 10% polyacrylamide gel containing 0.5% gelatin (50 mg ml⁻¹). Afterward gels were washed twice in 2.5% Triton X-100 for 30 min and incubated for 24 h at 37°C in 0.2 mol l⁻¹ NaCl, 5 mmol l⁻¹ CaCl₂, 0.2% Brij 35 and 50 mmol l⁻¹ Tris, pH 7.5. The buffer was decanted and the gels stained with Coomassie Blue followed by destaining. Identification of each gelatinase band was done in accord-ance to their molecular weight and commercial standards.

APS experimental model treatment with IVIG

Induction of fetal loss by passive transfer of anti-β2GPI antibodies and treatment. BALB/c mouse female (10–12 weeks old) and male mice (12–14 weeks old) were pur-chased from Tel-Aviv University and were mated. After confirming vaginal plugs, the mice were infused with anti-β2GPI (20 mg in 200 ml per mouse) through the tail vein on day 0 of pregnancy in order to induce fetal loss (9–11). On day 16 of pregnancy the mice were analyzed for fecundity, fetal resorptions and number of embryos.

The anti-β2GPI-infused group was divided in to five subgroups. The first left untreated, the second was treated with high dose (HD) of commercial IVIG (IVIG 400 mg kg⁻¹ 12 mg per mouse), the third was treated with low dose (LD) of commercial IVIG (IVIG 2 mg kg⁻¹ 60 μg per mouse), the forth was treated with sIVIG (sIVIG 2 mg kg⁻¹ 60 μg per mouse) and the fifth was treated with control IgG (400 mg kg⁻¹ 12 mg per mouse) through the vein on day 1 from the vaginal plug formation. Each group contained 50 mice.

Results

Fractionation and in vitro characterization of IVIG specific for APS (sIVIG) from a polyclonal preparation of IVIG

The anti-anti-β2GPI IVIG (sIVIG) was fractionated from a commercial compound of IVIG on an anti-β2GPI column. The anti-idiotypic activity of the sIVIG was tested by ELISA by in-

trouducing biotinylated sIVIG, commercial IVIG to anti-β2GPI bound to anti-Fc-coated ELISA plates. sIVIG bound signifi-cantly (P < 0.001) to anti-β2GPI antibodies and treatment.

APS experimental model treatment with IVIG

Induction of fetal loss by passive transfer of anti-β2GPI antibodies and treatment. BALB/c mouse female (10–12 weeks old) and male mice (12–14 weeks old) were pur-chased from Tel-Aviv University and were mated. After confirming vaginal plugs, the mice were infused with anti-β2GPI (20 mg in 200 ml per mouse) through the tail vein on day 0 of pregnancy in order to induce fetal loss (9–11). On day 16 of pregnancy the mice were analyzed for fecundity, fetal resorptions and number of embryos.

The anti-β2GPI-infused group was divided in to five sub-

groups. The first left untreated, the second was treated with high dose (HD) of commercial IVIG (IVIG 400 mg kg⁻¹ 12 mg per mouse), the third was treated with low dose (LD) of commercial IVIG (IVIG 2 mg kg⁻¹ 60 μg per mouse), the forth was treated with sIVIG (sIVIG 2 mg kg⁻¹ 60 μg per mouse) and the fifth was treated with control IgG (400 mg kg⁻¹ 12 mg per mouse) through the vein on day 1 from the vaginal plug formation. Each group contained 50 mice.
a dose-dependent manner (Fig. 1a). Similarly, sIVIG significantly inhibited the binding of anti-β2GPI affinity purified from five different APS patients, \( P < 0.001 \), (Fig. 1b). Moreover, sIVIG was significantly more efficient than the commercial IVIG in inhibiting the β2GPI binding by the preparations of anti-β2GPI antibodies from five patients, 76% inhibition with sIVIG in comparison to 22% inhibition at 50 mg ml\(^{-1}\) (Fig. 1b).

The effect of anti-anti-β2GPI-sIVIG on reproductive failure in naive mice

Pregnancy impairment was induced in BALB/c mice by passive transfer of a mix of anti-β2GPI antibodies, affinity purified from five patients with APS at day 0 of pregnancy and treated with sIVIG or commercial IVIG. Fetal loss and the fecundity were followed in all groups of mice. The anti-β2GPI-infused mice showed reduced pregnancy outcome than the control BALB/c as shown in Table 1: 26.5% and 53.6%, respectively, \( P < 0.02 \). Administration of sIVIG increased the fecundity to 46.43% in the anti-β2GPI-infused mice, while commercial IVIG at low concentration as the sIVIG did not have any significant effect \( P > 0.05 \). Similar to the effect of sIVIG on mouse fecundity, improvement in fetal loss was observed as well in anti-β2GPI-infused mice following therapy with LD sIVIG (2 mg kg\(^{-1}\)) and HD IVIG (400 mg kg\(^{-1}\)), \( P < 0.02 \). Therefore, we can conclude that sIVIG was 200 times more effective in repairing the fecundity of the BALB/c anti-β2GPI-infused mice. LD commercial IVIG did not have any significant effect on fetal loss, \( P > 0.05 \).

The effect of sIVIG on in vitro model of implantation

Based on the low pregnancy outcome following passive transfer of anti-β2GPI in BALB/c matted mice and the improved fecundity following treatment with sIVIG, the possible effect of IVIG on the trophoblast implantation process was assessed. The in vitro double-chamber invasion assay gave us a tool for studying a model of implantation. We used the JAR choriocarcinoma cells and confirmed the results using human 8–9 weeks old trophoblast cells.

Employing the matrigel system found that anti-β2GPI inhibited significantly the in vitro choriocarcinoma cell invasion in a dose-dependent manner (Fig. 2). For example, 9.5 ± 0.4% of invasion was documented with 50 mg ml\(^{-1}\) of anti-β2GPI in comparison to 37.3 ± 2.5% of invasion in the presence of IgG originated from a healthy individual at the same concentration \( P < 0.03 \) 39% inhibition of invasion (Fig. 2). The biological function of the anti-β2GPI antibodies is related to the β2GPI-binding site of the Ig since a mixture of β2GPI-related synthetic peptides, previously shown to

### Table 1. The effect of sIVIG on pregnancy outcome induced by anti-β2GPI antibodies

<table>
<thead>
<tr>
<th>Ig administration</th>
<th>Treatment</th>
<th>Fecundity (%)</th>
<th>Fetal resorptions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μg per mouse iv</td>
<td>Anti-β2GPI Non</td>
<td>26.5</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>Anti-β2GPI HD—IVIG 400 mg kg(^{-1}) 12 mg per mouse</td>
<td>38.7</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Anti-β2GPI LD—sIVIG 2 mg kg(^{-1}) 60 μg per mouse</td>
<td>46.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Anti-β2GPI LD—IVIG 2 mg kg(^{-1}) 60 μg per mouse</td>
<td>24.2</td>
<td>63.6</td>
</tr>
<tr>
<td></td>
<td>IgG control Non</td>
<td>53.6</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Non No treatment</td>
<td>55.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
neutralize anti-β2GPI activity (11), prevented the anti-trophoblast invasive properties of the antibodies in a dose-dependent manner (Fig. 3). For example, 10 mg ml⁻¹ β2GPI-related synthetic peptides reduced the percent invasion of choriocarcinoma from 36.6 ± 4.1 to 14.2 ± 1.2%, equivalent to 38.8% inhibition of invasion (Fig. 3).

Addressing the effect of sIVIG on the anti-β2GPI-induced inhibition of trophoblast cell invasion, we observed the following data as described in Fig. 4: pre-incubation of affinity-purified anti-β2GPI 10 mg ml⁻¹ with IVIG or F(ab)₂ portion of IVIG (28 mg ml⁻¹) improved the trophoblast cell invasion (P < 0.02). Fc fraction of IVIG did not affect the anti-invasive properties of anti-β2GPI (P > 0.05). Neutralizing the anti-β2GPI-binding site by β2GPI-related synthetic peptides (directed to the Fab of the Ig) prevented significantly the IVIG effect on the anti-invasive activity of anti-β2GPI antibodies (P < 0.01), (Fig. 4). Interestingly, when the trophoblastic cells were pre-incubated with the β2GPI-related synthetic peptides alone, we could see a decrease in the anti-invasive properties (P < 0.001).

The effect of sIVIG on trophoblast invasive properties is described in Fig. 5. Anti-β2GPI antibodies inhibited significantly (P < 0.02) the invasion of human trophoblastic cells (Fig. 5a). Pre-incubation of anti-β2GPI with sIVIG at 50 mg ml⁻¹ or HD of commercial IVIG (28 mg ml⁻¹) neutralized the anti-β2GPI activity and improved significantly (P < 0.02) the invasive properties of the human trophoblastic cells. LD IVIG at equal concentration as sIVIG did not affect the anti-β2GPI-mediated inhibitory effect (P > 0.05). HD IVIG by itself as well as sIVIG did not affect directly trophoblast invasion at the studied concentrations (P > 0.05).

As described in Fig. 5(b), subjection of sIVIG or IVIG in concert with anti-β2GPI to trophoblast invasion assay resulted in a dose-dependent inhibitory effect on anti-β2GPI-mediated defective trophoblast invasiveness. sIVIG was 560 times more effective than native form of IVIG (P < 0.001) (Fig. 5b). LD of sIVIG (50 mg ml⁻¹) had similar effect as HD IVIG (28 mg ml⁻¹).

**sIVIG effect on MMP2 and MMP9 secretion by trophoblast in the presence of anti-β2GPI antibodies in a trophoblast invasiveness assay**

Analysis of the culture fluid of all the trophoblast invasion assays for the presence of MMP2 and MMP9 revealed that anti-β2GPI inhibited the secretion of MMP2 and MMP9 by human trophoblast cells as shown in a representative Fig. 6 (lane A: MMP secretion by trophoblast cells with no treatment, lane B: MMP secretion upon exposure of trophoblast cells to anti-β2GPI). sIVIG and HD IVIG neutralized the anti-β2GPI-induced inhibition of MMP2 and MMP9 secretion as shown in lanes C and D, respectively. LD of IVIG, equivalent to the sIVIG dose, had no effect on anti-β2GPI-induced inhibitory effect on MMP2 and MMP9 secretion, lane E. HD of c-IgG from healthy individual (lane F). HD of IVIG lane G and LD IVIG lane H did not have any direct effect on the trophoblast MMP2 and MMP9 secretion.

**Discussion**

Our results show for the first time the beneficial activity of fractionated specific IVIG, namely anti-anti-β2GPI specific IVIG (sIVIG), for anti-phospholipid related reproductive failure. This fraction was affinity purified from an IVIG preparation, on a column composed of anti-β2GPI antibodies affinity purified from 15 APS patients. Infusion of the polyclonal anti-β2GPI antibodies into naive mice reduced the fecundity of the mice and enhanced the percentage of fetal loss. Administration of APS specific IVIG to anti-β2GPI-induced-APS mice, was
200 times more efficient in repairing the fecundity and fetal loss than the original IVIG.

The proof that IVIG encompass anti-anti-cardiolipin-β2GPI-dependent anti-idiotypic antibodies is based on the following studies: (i) IVIG, specifically the F(ab)2, ameliorate the Lupus anti coagulant (LAC) and anti-phospholipid antibodies activities (32, 33); (ii) IVIG reduced in vivo induction of APS experimental model by passive transfer of human anti-β2GPI idiotype (33). Moreover, administration of monoclonal anti-anti-cardiolipin-β2GPI-dependent anti-idiotypic antibodies into experimental APS mice induced by anti-β2GPI human mAb abrogated the generation of mouse anti-β2GPI antibodies as well as fetal loss and other APS clinical features (34).

In order to analyze the mechanisms by which sIVIG is superior to the whole IVIG compound, we employed the in vitro trophoblast invasiveness assay resembling the in vivo implantation process and analyzed the mechanism by which the beneficial activity of sIVIG may regulate the implantation process. Previously, it was reported that anti-β2GPI antibodies inhibit trophoblast invasiveness in vitro (35–37). Several mechanisms were proposed to explain the effect of anti-β2GPI on trophoblast such as inhibition of gonadotropin secretion (35, 36) and reduction in the Bcl-2/Bax ratio, without any clear apoptotic effect on the level of protein and mRNA (37). We add in the current study also the ability of anti-β2GPI to inhibit MMP2/MMP9 secretion by trophoblast cells, which are crucial for successful implantation, as an additional mechanism.

APS-specific sIVIG in an in vitro trophoblast invasion assay was significantly more competent in preventing trophoblast invasion of invasiveness induced by anti-β2GPI antibodies in vitro as defined by proliferation assays. We demonstrated herein that anti-β2GPI antibodies affect the trophoblast function in vitro via its Fab portion of the molecule since β2GPI-related synthetic peptides directed to the

![Fig. 4.](https://academic.oup.com/intimm/article-abstract/19/7/857/698703) IVIG F(ab)2 and Fc fragments effect on in vitro trophoblast cell invasion. Trophoblast invasiveness was analyzed in the presence of IVIG or its F(ab)2 and Fc derivatives and anti-β2GPI antibodies. The data are presented as mean ± SD of three different experiments.

![Fig. 5.](https://academic.oup.com/intimm/article-abstract/19/7/857/698703) (a) The effect of IVIG, sIVIG on in vitro human trophoblastic cells invasion. Inhibition of trophoblastic cell invasion in a matrigel invasion system was induced by anti-β2GPI antibodies. sIVIG (10 mg ml⁻¹) was compared with commercial IVIG LD (10 mg ml⁻¹) and HD (28 mg ml⁻¹) for neutralization of anti-β2GPI effect. (b) The efficacy of sIVIG, IVIG on in vitro human trophoblastic cells invasion dose response. Dose-dependent effect of sIVIG and IVIG on the anti-β2GPI-induced trophoblast anti-invasive properties. C-IgG, a control IgG from one individual had no effect on trophoblastic cell invasion, was used as a negative control, (upper dashed line). The anti-β2GPI anti-invasive effect is shown in the lower dashed line. The data are presented as mean ± SD of three different experiments.

![Fig. 6.](https://academic.oup.com/intimm/article-abstract/19/7/857/698703) The effect of sIVIG on MM2, MMP9 secretion by human trophoblastic cells. Gelatinase activity detected in the trophoblast invasion assay culture fluid. MMP2 and MMP9 activity: lane A: MMP secretion by trophoblast cells with no treatment, lane B: MMP secretion upon exposure trophoblast cells to anti-β2GPI. Lanes C and D, respectively, sIVIG and HD IVIG. Lane E: LD of IVIG. Lane F: HD of IVIG. Lane G: sIVIG and lane H did not have any direct effect on the trophoblast MMP2 and MMP9 secretion.
antigen-binding site of the antibody molecule neutralized its binding to trophoblast and abrogated trophoblast invasiveness. However, incubation of trophoblast cells with a cocktail of β2-GPI-related synthetic peptides inhibited trophoblast invasiveness. This fact could be attributed to the peptide 274GDKVSFFC1397KEKKC388 which has a dual function; on one hand it is a target molecule for subpopulation of anti-β2-GPI antibodies and on the other hand it is the phospholipid-binding site which binds the trophoblast (i.e. rich in phosphatidylserine) (29, 38). Thus, the binding of this fifth domain peptide to the trophoblast may prevent the β2-GPI binding. As was demonstrated before in β2-GPI knockout mice lacking β2-GPI, the β2-GPI molecule is required for successful implantation and placental morphogenesis (39). The ability of β2-GPI-related peptides to neutralize the Fab of the anti-β2-GPI, thus reversing the anti-β2-GPI-mediated inhibition of trophoblast invasiveness, support the importance of anti-β2-GPI neutralization by IVIG for normal trophoblast invasion. Indeed, APS-specific IVIG—sIVIG had 560 times more beneficial effect on trophoblast invasiveness properties. Furthermore, we propose herein that one of the mechanisms affected by anti-β2-GPI is the down-regulation of the MMP2 and MMP9 secretion by trophoblast cells, which could be enhanced by sIVIG treatment or by HD IVIG. MMPs, a family of endopeptidases with the ability to degrade extracellular matrices (ECM) proteins, play a fundamental role in inflammation, tissue remodeling, angiogenesis, wound healing, tumor invasion and metastatic progression (31). Among the MMPs, MMP2 and MMP9 are key enzymes synthesized as latent proenzymes, which must be activated in order to show their proteolytic activities and degrade various components of the ECM including type IV, V, VII and X collagens, fibronectin and gelatin (40, 41). MMP2 and MMP9 have been extensively investigated because of their recognized roles in early pregnancy (31–37). It has been demonstrated that MMP2 and MMP9 play key roles in ECM degradation and trophoblast invasion during early pregnancy and are highly expressed during implantation and early stage of pregnancy (42, 43). We have shown herein for the first time that anti-β2-GPI affect trophoblastic invasiveness by inhibiting MMP9 and MMP2 secretion. HD IVIG and LD sIVIG were able to elevate the MMP2 and MMP9 secretion by trophoblast cells by neutralizing the anti-β2-GPI inhibitory activity on trophoblast MMP’s secretion. IVIG and sIVIG had no direct effect on trophoblast MMP’s secretion.

In the past, several potential mechanisms were proposed to explain these APS-related obstetric complications: (i) the requirement for complement activation in vivo for anti-phospholipase-induced fetal loss was documented (13, 44). Inhibition of the complement cascade in vivo, using the C3 convertase inhibitor complement receptor 1-related gene/protein (Crry)-Ig, blocked fetal loss and growth retardation, a phenomenon currently shown also in complement C3-deficient mice (13); (ii) Anti-β2-GPI-mediated endothelial activation leading to induction of adhesion molecule expression including E-selectin, ICAM-I and vascular cell adhesion molecule-I, associated with elevated expression of NFκB, MyD88 and involvement of p38 MAP-kinase in the up-regulation of tissue factor on endothelial cells (12, 45–47); (iii) Anti-β2-GPI antibodies mediated inhibition of HCG secretion in an ex vivo placental model, as well as in vitro trophoblast invasiveness model (34, 35) and (iv) Anti-β2-GPI inhibit placental differentiation (48). Anti-β2-GPI may impair placenta by increasing apoptosis and attenuating mitosis of trophoblast cells (49). IVIG is known to have immunomodulatory effect on apoptotic process. On one hand it may exert anti-apoptotic properties or pro-surviving function like in pemphigus vulgaris IgGs induced acantholysis, in which IVIG protect target cells from apoptosis by up-regulating endogenous caspase and calpain inhibitors (50). Alternatively, IVIG is known to cause anti-glycoprotein-IIb-induced platelet apoptosis in a murine model of immune thrombocytopenia or may induce apoptotic processes like in human B cells (51, 52). Other group showed that Fc-gamma receptor-independent mechanism is involved in an inhibitory effect of IVIG on the thrombogenic effects of anti-phospholipid in ex vivo model of thrombosis and endothelial cells (53). The trophoblast Fc receptor is Fc-n (neonatal Fc-receptor) and is not involved in the current set of in vitro experiments since control IgG had no effect on the trophoblast function.

In summary, the results of this study support the idea that similar to what has been shown in other humoral-mediated autoimmune diseases, the beneficial effect of IVIG in APS—particularly in pregnancy loss—is due to the presence of specific anti-idiotypic antibodies. Here, we purified specific anti-idiotypic antibodies and demonstrated superior efficiency in a mouse model of APS in comparison to total IVIG. We propose that LD sIVIG and HD IVIG abrogate trophoblast invasion inhibition mediated by anti-β2-GPI antibodies, involving neutralization of the anti-β2-GPI activity and elevation of MMP9 and MMP2 secretion. Our study underscores the possibility to fractionate many specific anti-idiotypic IgG from the same batch of IVIG that may then be utilized in various autoimmune conditions.

We have to keep in mind that different preparation of IVIG may contain different amounts of anti-idiotypic antibodies due to the differences in the population that IVIG was derived from.

Acknowledgements
This work was supported by AUTOROME European Community grant no. LSHM-CT-2004-005264.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>anti-phospholipid syndrome</td>
</tr>
<tr>
<td>β2-GPI</td>
<td>beta-2-glycoprotein-I</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrices</td>
</tr>
<tr>
<td>HD</td>
<td>high dose</td>
</tr>
<tr>
<td>IVIG</td>
<td>intravenous immunoglobulin</td>
</tr>
<tr>
<td>LD</td>
<td>low dose</td>
</tr>
<tr>
<td>MMP</td>
<td>metalloproteinase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>sIVIG</td>
<td>specific intravenous Ig</td>
</tr>
</tbody>
</table>

References

Specific IVIG in experimental antiphospholipid syndrome


