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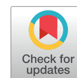
Method Article

Methodology of generation and purification of anti-beta 2 glycoprotein I antibodies



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A B S T R A C T

In this Method Article we are showing the methodology for generation and purification of Anti-Beta 2 Glycoprotein I (β_2GPI) antibodies. First β_2GPI was purified from human plasma, and recognized by Western Blot and anti-β_2GPI antibodies of serum from patients with antiphospholipid syndrome (APS). The C57BL/6 mice were immunized intraperitonealy with 150 μg of protein in adjuvant (β_2GPI or bovine serum albumin) on days 1, 8 and 14. Then the anti-β_2GPI antibodies were purified by affinity chromatography (Affi-Gel protein A sepharose) and affinity column using human β_2GPI coupled to CNBr-activated Sepharose 4B. Titles of anti-β_2GPI antibodies were determined by ELISA assays.

- We purified β_2GPI with great efficacy and that is recognized antigenically by serum from patients with SAP or an anti-$\beta_2$gpi antibody.
- We found that our purified antibody had 13 fold increased activity in ELISA test compared with the control and in Western Blot recognized with β_2GPI (reference and purified).

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Specifications Table

Subject Area:	*Immunology and Microbiology*
More specific subject area:	*Immunology*
Method name:	*Generation and purification of Anti-Beta 2 Glycoprotein I antibodies.*
Name and reference of original method	*Pierangeli, S.S., and Harris, E.N. 1993. Induction of phospholipid-binding antibodies in mice and rabbits by immunization with human beta 2 glycoprotein 1 or anticardiolipin antibodies alone. Clin. Exp. Immunol. 93:269-272.*
Resource availability	*NA*

Method details

Purification of β_2GPI

Human β_2GPI was purified according to previously described methods [1], with some modifications. Briefly, perchloric acid (70%) was added to outdated pooled plasma to a proportion 1:33 (acid:plasma), stirred for 30 min at 4 °C and then centrifuged at 13,000 g for 15 min at 4 °C. The precipitate was discarded and the supernatant was adjusted to pH 7.4 with NaOH 5 M, 43 g of ammonium sulfate powder was added to 100 mL of supernatant, and the mixture was stirred at 4 °C for 30 min. After centrifugation at 13,000 g for 15 min at 4 °C, the precipitate was dissolved in 30 mM NaCl, 20 mM Tris-HCl, pH 8.0, and followed by extensive dialysis against the same buffer by 24 h with buffer changes every 6 h. The dialysate was applied to a column of Heparin-Agarose (Sigma-Aldrich, MO, USA) and then incubated by 30 min. and washed sequentially with 50 mM NaCl, 20 mM Tris, pH 8.0, and 150 mM NaCl, 20 mM Tris, pH 8.0, and then eluted with 350 mM NaCl, 20 mM Tris, pH 8.0 and collected of to 1 ml. Then was measured at 280 nm. The peaks (containing β_2GPI) were collected, concentrated and dialyzed against PBS buffer pH 7.4. Finally, the purity was verified in a Coomasie Blue stain and Western Blot, also the capacity was determined by three patient serum with APS (Donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch) in ELISA assays, the fraction was maintained at −80 °C. As a positive control for ELISA and Western Blot, a reference β_2GPI was used donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch.

Immunization protocol

Immunization was according to previously described methods [2]. Eleven C57BL/6 mice (five from each group) were immunized intraperitonealy with 150 μg of protein in adjuvant (β_2GPI or bovine serum albumin, BSA) on days 1, 8 and 14. All mice were sacrificed after day 28. Blood samples were drawn and the titles of anti-β_2GPI antibodies were determined by ELISA assays.

Protein a purification of IgG

The plasma positive for anti-β_2GPI antibodies of all immunized mice with β_2GPI were pooled and whole IgG was purified using Affi-Gel Protein A MAPS II Kit (Bio-Rad, CA, USA). The affinity of various IgG subclasses, especially from APS, are higher for Protein A than for Protein G [3,4]. Briefly, the column was equilibrated with binding buffer (pH 9.0), the pooled plasma was applied to the column of protein A that was washed with binding buffer and the IgG was eluted with elution buffer (pH 3.0), collected of 1 ml and neutralized immediately with saturated solution of Na_2CO_3. The absorbance was reader to 280 nm, the peaks (containing IgG) were collected, concentrated and dialyzed against PBS buffer pH 7.4, and the fraction was maintained at −80 °C. The IgA and IgM data were not included since IgG is the one with the highest clinical correlation in APS [5].

Isolation of IgG anti-β_2GPI antibodies

The isolation was according to previously described methods [6]. The β_2GPI previously purified was coupled to Cyanogen bromide-activated Agarose (Sigma-Aldrich, MO, USA). Briefly, 1 g of the CNBr activated agarose was mixed with 1 mM HCl, then 15 mg of pure unnicked β_2GPI dissolved in 0.1 M NaHCO3/0.5 M NaCl, pH 8.3 was binding to 4.0 ml of the activated agarose. The solution was stirred at 4 °C over night. The unreacted sites were blocked with 0.2 M glycine pH 8.3. Finally it was washed 10 times alternating with 0.1 M NaHCO3/0.5 M NaCl, pH 8.3 and 0.1 M acetate buffer/0.5 M NaCl, pH 4, loaded to the column, equilibrated by PBS pH 7.4 and stored at 4 °C. The IgG fraction from mice pooled plasma that was isolated with Affi-Gel Protein A MAPS II Kit (Bio-Rad, CA, USA) was applied to the CNBr-β_2GPI column. After washing with the same buffer, bound anti-β_2GPI antibodies were eluted with 0.1 M glycine-HCl pH 2.5. Eluates were collected from 1 ml and neutralized immediately with saturated solution of Na_2CO_3. The absorbance was reader to 280 nm, the peaks (containing anti-β_2GPI antibodies) were collected, concentrated and dialyzed against PBS buffer pH 7.4. Finally, we analyzed the capacity to react of this antibody with two different β_2GPI (reference and purified) by ELISA and Western Blot, the fraction was maintained at −80 °C.

Anti-β_2GPI antibodies ELISA

The IgG anti-β_2GPI antibodies were determined by ELISA as previously described [7–9]. In brief, micro-titer plates (Maxisorp 269787; Thermo Scientific Nunc) were coated overnight to

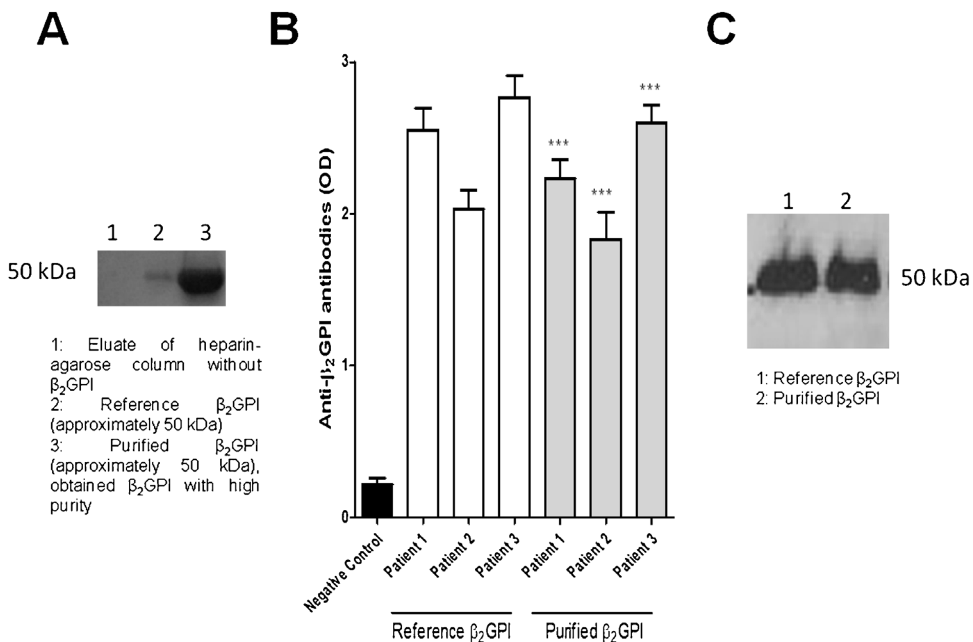

Fig. 1. Characterization of purified Beta 2 glycoprotein I. (A) Coomassie blue stain of β_2GPI, was isolated from normal human serum and applied to an SDS 8% polyacrylamide gel under nonreducing conditions. Line 1: Eluate of heparin-agarose column without β_2GPI; 2: reference β_2GPI (approximately 50 kDa) and 3: purified β_2GPI (approximately 50 kDa), obtained β_2GPI with high purity. (B) Was evaluated the reactivity of three patients serums with APS recognized with high specificity both β_2GPI the reference and purified in ELISA plate coated with these antigen, the bound were detected by an alkaline-phosphatase-labeled goat anti-human IgG antibody and error bars represent mean ± SEM of duplicate points. Negative control: healthy patient. (C) Western Blot of the same proteins shown in A using a reference anti-β_2GPI antibody. Line 1: reference β_2GPI and Line 2: purified β_2GPI. Both with similar molecular weight (approximately 50 kDa). ***$p < 0.001$ as compared with negative control.

4 °C with a solution containing 25 μg/mL of β_2GPI purified in the laboratory as was previously described or by β_2GPI reference (Donate by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch) and plates were blocked with 3% BSA (sufficient concentration to obtain a good blocking of the plate). Samples and controls (diluted 1:50 in 3% BSA solution) were added to the plates in triplicate and incubated by 1 h, pooled normal mouse plasma was used as negative controls. Plates were washed three times with PBS-Tween 20 0.5% pH 7.2 and incubated with alkaline-phosphatase anti-mouse IgG (Sigma-Aldrich, MO, USA). The color was developed by the addition of 1 mg/ml of *p*-nitrophenolphosphate (Sigma-Aldrich, MO, USA), and the reaction was stopped by the addition of 3 N NaOH. Then the plate was read in a Biotek ELx800 (Biotek, VT, USA) ELISA reader at 405 nm. A sample was considered positive when its OD was greater than 3 standard deviations (SD) from the average of normal controls (cut-off).

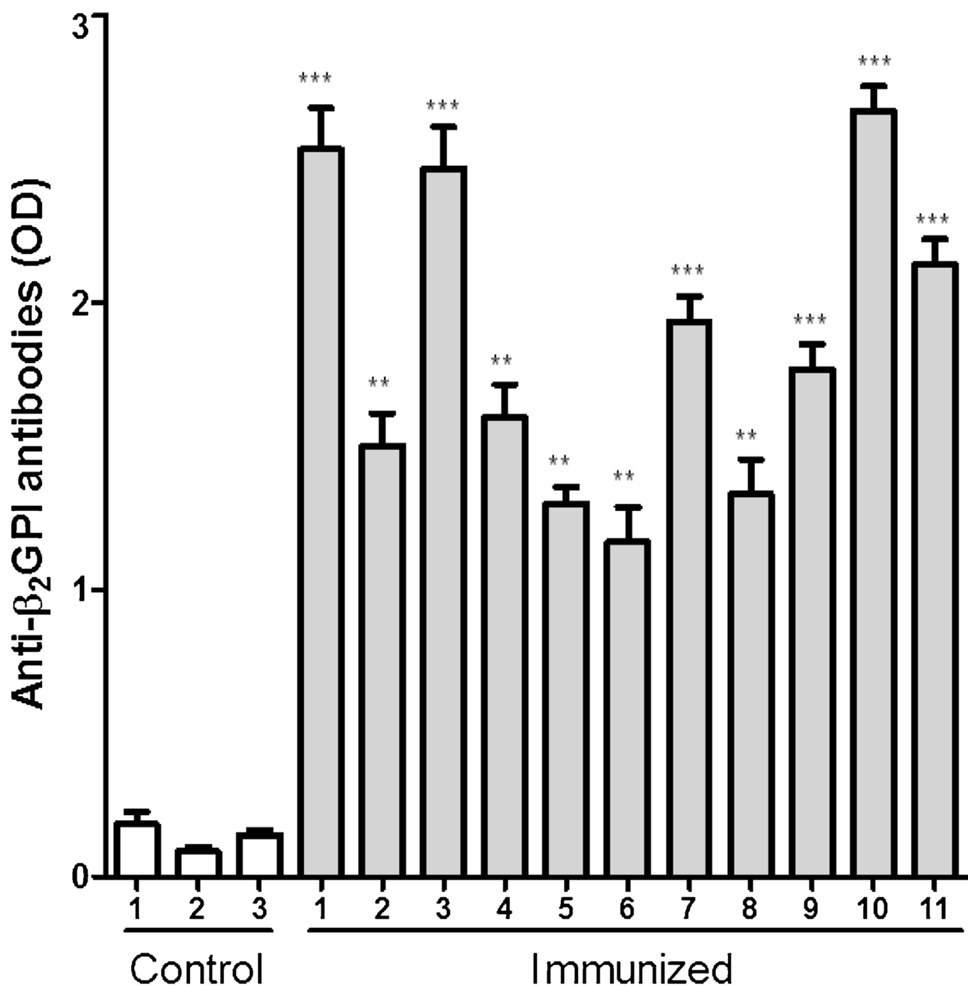

Fig. 2. Production of anti-β_2GPI antibodies in mice immunized with human β_2GPI. Eleven mice were immunized with three injection of 150 μg of human β_2GPI by 28 days and then the levels of antibodies were corroborate by ELISA assays detection, all mice shows high levels of anti-β_2GPI antibodies. Error bars represent mean ± SEM of duplicate points. **$p < 0.01$ and ***$p < 0.001$ as compared with control.

Western blot analysis of β_2-GPI expression

The β_2GPI (reference or purified, 10 ug) were mixed in lysis buffer (20 mm HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mm NaF, 1 mm phenylmethylsulphonyl fluoride, 10 μg of leupeptin per ml) and separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and then probed with polyclonal rabbit anti-human β_2GPI (Donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch). Afterwards, membranes were incubated with HRP-conjugated goat anti-rabbit IgG, and peroxidase reaction was visualized by the Pierce™ ECL Western Blotting Substrate (Thermo Scientific, MA, USA).

First purification protocol previously used by Thiagarajan et al. [1] with modifications was selected and the β_2GPI was isolated from human plasma (100 mL). After obtaining the pool fraction, the proteins were precipitated using ammonium sulfate solution and subsequent dissolution with 0.03 mol/L, NaCl 0.02 mol/L and Tris-HCl, pH 8.0. This solution was added to the heparin-agarose column and then was eluted with a solution of 0.35 M NaCl, 0.02 M Tris pH 7.2 and 1 M NaCl. Then in order to see the purity of β_2GPI, this was proceeded by PAGE-SDS and Coomasie blue stain (Fig. 1A), the protein electrophoresis showed major bands of 50 kDa (Fig. 1A, Line 3) to compare with a reference (Donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch) and

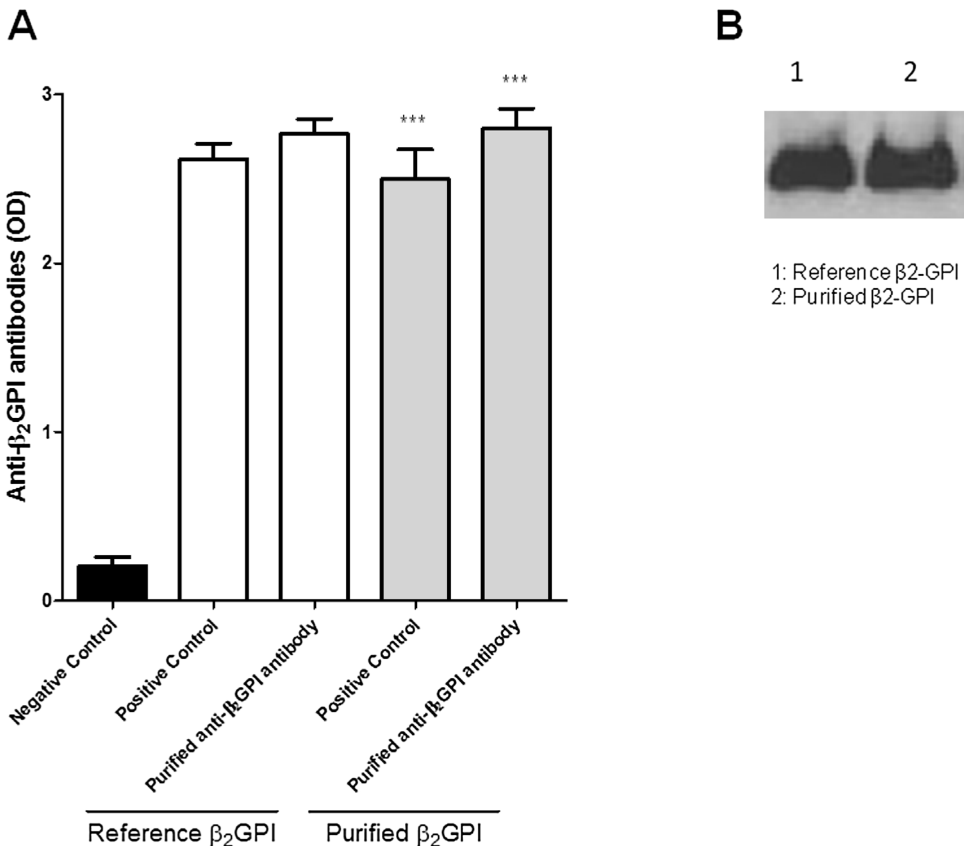

Fig. 3. Purified anti-β_2-GPI antibodies recognize to the β_2-GPI. The antibodies was purified by two column Affinity-purified type A IgG antibodies and CNBr-β_2GPI, we detected: (A) In ELISA the values of the anti-β_2-GPI antibodies exceeds for 13 folds to negative control (p 0.001) and positive control (patient 3 with APS), negative control: healthy patient, positive control: patients with APS (reactivity of cardiolipin and β_2GPI), error bars represent mean ± SEM of duplicate points or (B) In Western Blot show high reactivity with reference β_2-GPI (Line 1) or purified β_2-GPI (Line 2). ***$p < 0.001$ as compared with negative control.

β_2GPI was confirmed (Fig. 1A). For confirmation β_2GPI purified, serum from three patients with APS (with reactivity of cardiolipin and β_2GPI) was used (Donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch, with high levels of antibodies >80 GPL) by ELISA, all patients shown were highly reactive with reference β_2GPI and β_2GPI purified and the negative control (healthy patient) (Fig. 1B). Finally through Western Blot the capacity of purified β_2GPI was recognized by an anti-β_2GPI antibody (Donated by S.S. Pierangeli PhD, Division of Rheumatology University of Texas Medical Branch), a similar result was found that both β_2GPI (reference and purified) were detected (Fig. 1C). Therefore, we purified β_2GPI with great efficacy and that is recognized antigenically for serum from patients with APS or an anti-β_2GPI antibody.

Eleven mice were immunized with human β_2GPI (150 μg/mL) and three control mice with human albumin (an irrelevant protein antigens, 150 μg/mL), with three intra-peritoneal injections within a total of 28 days. To determine whether plasma from immunized mice had generated anti-β_2GPI antibodies and these recognizing the three-dimensional structure of purified protein ELISA assays were performed (the antibodies that detected were of the only IgG isotype), which manifested the presence of specific antibodies and the immunization produced high levels of antibodies (Fig. 2) in all mice. According to the obtained results the immunized mice were 13 fold increased (1.855 ± 0.160) compared with control mice (0.139 ± 0.0028) in the production of antibodies. All controls do not present positivity for anti-β_2GPI antibodies. Then the anti-β_2GPI antibodies was purified, first it was proceeded to obtain the antibodies only class IgG, for that all serum of immunized mice were pooled and purified by Affinity chromatography used Affi-Gel protein A sepharose were eluted and the fractions mixed the column with high peaks of absorbance. Secondly the anti-β_2GPI antibodies was purified by affinity column using human β_2GPI coupled to CNBr-activated Sepharose 4B, like the previous column the peaks were combined and concentered. The avidity of these antibodies was determined by ELISA assays and Western Blot (Fig. 3), we found that our purified antibody had 13 fold increased activity in ELISA compared with the control and in Western Blot recognized with both β_2GPI (reference and purified; negative control healthy patient and positive control APS patient). Despite the absence of purity data, there was no significant difference in the OD values between purified anti-beta2GPI antibodies and patient's serum with APS. Data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and expressed as mean ± standard error of mean (SEM). Three or more independent experiments were performed for the different assays. Differences between groups were analyzed by Student's *t*-test. P-values <0.05 were considered significant.

Supplementary material and/or additional information

NA.

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