Detection of IgG anti-beta2 glycoprotein-I antibodies in Saudi patients with systemic lupus erythematosis

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ABSTRACT

الأهداف: يعد مرض الذأب الاحمراري الجهازي من الأمراض ذاتية المناعة الشائعة لدى النساء وتهدف الدراسة إلى مقارنة مستويات الأجسام المضادة لبيتا2– لدى مرضى الذأب الاحمراري الجهازي.

الطريقة: أجريت هذه الدراسة في المنطقة الشرقية من السعودية بين عامي 2004م و 2006م، حيث تمت الدراسة على 50 مريضاً سعودياً مصابون بالذأب الاحمراري الجهازي و 50 شخصاً متبرعاً (مجموعة التحكم) وقد تم تطوير وتصميم تقنية محدثة من (ELISA) لقياس مستويات الأضداد لديهم.

النتائج: أظهرت الدراسة ارتفاع في الأجسام المضادة لبيتا2– لدى 80% من مرضى الذأب الاحمراري الجهازي، كما لوحظ وجود علاقة طردية بين ارتفاع مستوى الأضداد وكل من تجلط الدم و نقص الصفائح الدموية.

خاتمة: كشفت الدراسة إن ارتفاعاً في الأجسام المضادة لبيتا2– لدى 80% من مرضى الذأب الاحمراري الجهازي يمكن استخدامه كعامل تنبؤ بالمضاعفات المستقبلية، لكننا بحاجة الى دراسات إضافية لتأكيد أي علاقة قد توجد بين ارتفاع مستوى الأضداد وأي أعراض سريرية.

Objectives: To develop an assay for the measurement of this anti-human β2-glycoprotein I (aβ2-GPI)

Methods: This study was conducted from September 2004 to December 2006. The patients attending the Rheumatology Clinic were chosen from several centers in the Eastern region of Saudi Arabia because they had complications. An enzyme-linked immunosorbent (ELISA) assay was optimized and developed to measure IgG aβ2-GPI antibody levels in humans. Fifty normal blood donors and 50 systemic lupus erythrematosis (SLE) patients were selected for this experiment.

Results: Raised IgG aβ2-GPI antibody levels were found in 80% of SLE patients. Interestingly, raised IgG aβ2-GPI antibody levels were associated with the presence of venous thrombosis and thrombocytopenia.

Conclusion: The real value of IgG aβ2-GPI as a predictor for the future clinical complications needs to be confirmed in prospective controlled studies investigating clinical complications in relationship to IgG aβ2-GPI and to other risk factors for thrombosis.

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Human β2-glycoprotein I (β2-GPI) is a glycoprotein with a molecular weight of 50kD, which was first isolated in 1961.[1] It is associated with several plasma lipoprotein fractions and is also called apolipoprotein H (ApoH).[2] Lozier et al[3] found that β2-GPI is a single chain glycoprotein consisting of 326 amino acids residues. Beta2-GPI is present in normal plasma at a concentration of 150-300mg/l.[4] The protein is synthesized in the liver and also in the placenta. Studies by Polz et al[4] discovered that β2-GPI is a component of the protein part of many lipoproteins, such as very low density lipoproteins and high density lipoprotein. One of the main properties of β2-GPI is its ability to bind molecules bearing negatively charged groups including deoxyribonucleic acid (DNA), phospholipase, and heparin.[5,6] Most investigations on the physiological role of β2-GPI concern its involvement in the coagulation

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system.[7] It also inhibits adenosine diphosphate (ADP)-induced platelet aggregation. The serum concentration of β2-GPI is influenced by gender (higher in men), pregnancy (lower), age (higher in older people), liver cirrhosis (lower), and diabetes mellitus (higher), whereas, fasting, malignancy and rheumatoid arthritis do not affect its concentration.[8,9] Clinically, anti-human β2-glycoprotein I (aβ2-GPI) antibodies are found in association with anticardiolipin (aCL) antibody and lupus anticoagulant, and has been reported to be strongly associated with thrombosis and pregnancy loss.[10-12] Some patients with systemic lupus erythrematosis (SLE) have been described to have aβ2-GPI but not aCL.[13-16] Systemic lupus erythrematosis is a chronic multiorgan disorder affecting predominantly young women (20-45 years). Clincopatholgic features are diverse and virtually any organ, tissue, or system can be involved. The clinical signs include: malaise, fever, butterfly rash, eruptions, glomerulonephritis, depression, and weight loss.[17] This disease is associated with a consistent feature, which is the development of antinuclear antibodies, particularly against DNA. Other autoantibodies against the cytoplasmic component may also be present.[18,19] Several studies investigated the prevalence of aβ2-GPI antibodies in SLE patients. The results of these studies showed a wide variation in the prevalence of aβ2-GPI antibodies. The reasons for this are that aβ2-GPI enzyme-linked immunosorbent Assay (ELISA) has not yet been standardized and results from different studies have therefore been reported in different ways [raw optical density (OD) data, units, positive/negative], the cut-off levels for positivity was not the same for all studies, and the patient population differed from one study to another.[20-24] This study was designed to measure the aβ2-GPI in SLE Saudi patients. We also examined possible associations between increased levels of immunoglobulin G (IgG) aβ2-GPI and the clinical manifestations and evaluated the usefulness of IgG aβ2-GPI measurements in the diagnosis and management of SLE.

Methods. ***Characterization of the product.*** Immunoglobulin G aβ2-GPI was purified from human serum using a combination of precipitation with ethodin, sodium chloride (NaCl), followed by affinity chromatography on heparin-sepharose and protein G column. The purity of the final product was assessed by double radial immunodiffusion (RID), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, and ELISA study. The final product had a 50 kDa and an antigen used for the detection of aβ2-GPI was human β2-GPI. Human β2-glycoprotein I from human serum was purified using a modification of the method of Polz et al.[4] In order to optimize this ELISA, we tested different microtiter plates, antigen concentration, blocking, diluting solutions, sample dilution, and antibody conjugates, before adopting the final protocol of the assay. The optimized assay was used to measure the IgG aβ2-GPI antibody levels of 50 normal blood donors and 50 SLE patients.

Selection of patients. This study was conducted from September 2004 to December 2006. The patients attending the Rheumatology Clinic were chosen from several centers in the Eastern region of Saudi Arabia because they had complications. Fifty Saudi SLE patients (age: 19-83 years, 47 female and 3 male) and 50 normal blood female donors (age, 22-57 years; all females) were selected for this study. All SLE patients fulfilled the American College of Rheumatology revised criteria for the classification of SLE.[25] Inter- and intra-assay precision of the assay were acceptable. Written informed consent and Research Ethics Committee approval were obtained in all cases.

Statistical analysis. Statistical analysis of the results was performed using the statistical computer package GraphPad prism. We used Mann Whitney U test to compare the means of aβ2-GPI levels in SLE patients and in normal subjects. Fisher's exact test was used to compare the proportion of positives for aβ2-GPI in SLE patients and in normal subjects, and Spearman's rank correlation test to correlate aβ2-GPI levels in SLE patients.

Results. ***Characterization of human b2 glycoprotein I.*** Three different types of flat-bottomed microtiter plates were compared for their ability to bind β2-GPI as described, Immuno™ plates with MaxiSorp™ surface (Nunc, Denmark), Microplate microtiter plates (Greiner, Germany), and Immunlon® 2 horse blood agar (HB) (Dynex, United Kingdom). The HB plates showed the greatest difference between specific and non-specific binding, as well as giving a low background, and were used for subsequent experiments and in the final protocol. Various concentration of β2-GPI for coating the plates, showed that increasing the coating concentration increases the specific binding and leaves the non-specific binding unchanged. Due to the limited number of human β2-GPI purified, a coating concentration of aβ2-GPI antibodies of 1μg/well was used in the final protocol. Various agents were tried as blocking solutions. Whist casein did not block the remaining binding sites effectively, gelatine showed a somewhat erratic behavior. Bovine serum albumin (BSA) seemed to give the best results for the measurement of aβ2-GPI. A 5% solution of BSA was used in the final protocol. Two coating/blocking regimes seemed to be practicable, either to coat for 2 hours at room temperature and to block overnight at 4˚C, or to coat overnight at 4˚C and to block for 2 hours at room temperature the following day. The choice of coating/

blocking regime did not influence the ELISA results. Therefore, the coating/blocking for 2 hours regime was used in the final protocol.

Finally, different combinations of sample and conjugate dilution were investigated. Results showed that the curves for specific and non-specific binding run in parallel when the conjugate dilution is varied. Therefore, a change in conjugate dilution does not lead to a better specific/non-specific binding ratio. Using a less dilute sample gave a higher OD, but for practical reasons (limited amount of sample), only a 1:100 sample dilution could be afforded in the final protocol. Conjugate dilutions used in the final protocol was 1:1000. The purity of the final product was assessed, along with a commercial preparation (Behring), by SDS-PAGE analysis, which gave a band at 52 kDa non-reduced and at 55 kDa reduced. (**Figure 1**). The increase in the apparent molecular weight of β2-GPI under reducing conditions is well known and has been described for different methods of purifications.[20] The nature of the upper band (55 kDa) is difficult to explain. Its molecular weight and affinity to heparin may suggest that this is anti-thrombin (AT III), which in humans has the same serum concentration as β2-GPI. Another hypothesis as to the nature of the higher molecular weight bands may, therefore, be that during the purification step the β2-GPI breaks down, and that the upper bands represent dimerized breakdown products. This hypothesis may be consistent with the lower heparin affinity of the upper band.

Measurements of anti-β2 anti-glycoprotein-I antibodies. The results of the aβ2-GPI measurements were expressed as arbitrary ELISA unit (AEU), with a sample with a suitably high reading being assigned an arbitrary value of 100 AEU, and aβ2-GPI concentrations in the various samples were calculated using the following formula:

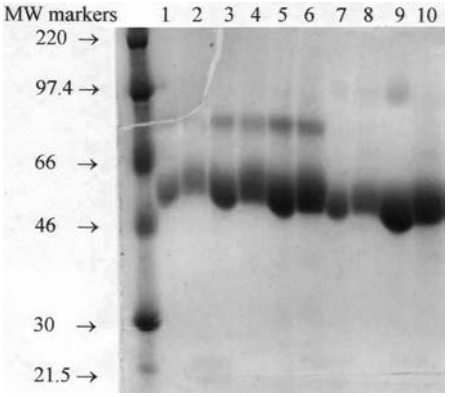

Figure 1 - Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of our final product (lanes 1 & 2 = 5mg, lanes 3 & 4 = 10mg and lanes 5 & 6 = 20mg), a commercial preparation (lanes 7 & 8 = 5mg, lanes 9 & 10 = 10mg). The first lane of each pair is non-reduced and the second lane is reduced.

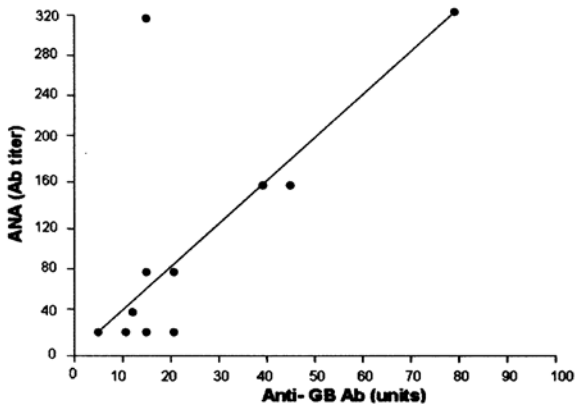

Figure 2 - Relationship between the concentration of ab2-GPI antibody and the ANA titer. Data obtained from 11 samples (p=0.001).

Table 1 - Clinical manifestations in systemic lupus erythrematosis (SLE) patients.

Manifestation	Frequency (n=50)
Arterial thrombosis	20
Venous thrombosis	10
Thrombocytopenia	5
Heart Valve disease	12
Any of the clinical manifestations	41

Table 2 - Levels of IgG anti-human β2-glycoprotein I (aβ2-GPI) levels in normal blood donors and systemic lupus erythrematosis (SLE) patients.

Levels	Normal blood donors	SLE patients
Range (in units)	0.06-12.5	0.02-150
Median	1.5	5.6
P-value		0.001

Table 3 - Clinical manifestations and IgG anti-human β2-glycoprotein I (aβ2-GPI) in systemic lupus erythrematosis (SLE) patients.

Manifestation	Relationship to IgG anti-β2 GPI (*p*-value)	No. of SLE patients n=21
Arterial thrombosis	0.009	6
Venous thrombosis	0.005	2
Thrombocytopenia	0.003	1
Heart valve disease	0.35	3
Any of the clinical manifestations	0.56	16

aβ2-GPI sample=OD sample÷OD standard×100 AEU The standard sample was measured in quadruplicate on each plate, and other samples in duplicate. The specific binding (namely measured in wells coated with β2-GPI) was subtracted from the non-specific binding (namely measured in wells coated with ethanol only). The distribution of aβ2-GPI levels was not normal, according to the Komolgorov-Smirnov test (KS distance 0.25, $p<0.0001$), and the normal range was therefore defined as the range of values which were lower than the 99th percentile namely, <12.5 arbitrary units (range 0-12.5 units). We also calculated the sensitivity, and specificity of aβ2-GPI testing for any of the investigated clinical manifestations, and we found that IgG aβ2-GPI had a sensitivity of 75% and a specificity of 91%. Of 50 patients, 41 patients presented with one or more of the clinical manifestations. The frequency of each manifestation in our SLE patients is shown in **Table 1**. The normal range for IgG aβ2-GPI was established by measuring the levels of these antibodies in serum samples from 50 normal blood donors. Levels of IgG aβ2-GPI ranged from 0.05 to 120 units in SLE patients. They were elevated in 46/50 of the SLE patients, while 3 patients were borderline. The statistical analysis of the IgG aβ2-GPI levels in both groups is presented in **Table 2**. Mean levels of IgG aβ2-GPI in SLE patients and in normal subjects were compared using Mann Whitney U test. The overall difference between the 2 groups was significant ($p=0.0001$).

Association of clinical manifestations and IgG b2 anti-glycoprotein-I antibodies. In order to examine the possibility of a pathogenic role for IgG aβ2-GPI. The association between elevated levels of IgG aβ2-GPI and the clinical manifestations were studied. The presence of the following manifestations was recorded in our patients: arterial thrombosis (stroke, transient ischemia attacks, myocardial infraction, peripheral arterial thrombosis), venous thrombosis (deep or superficial vein thrombosis, pulmonary hypertension), thrombocytopenia and heart valve diseases (thrombotic vegetations, valve dysfunction). Forty-one SLE patients have at least one of the above clinical manifestations. Using Fisher's exact test, we found that, for SLE patients, the clinical manifestations as a total were weakly associated to IgG aβ2-GPI ($p=0.56$). The association of elevated levels of IgG aβ2-GPI to specific clinical manifestations was also examined. In SLE patients, significant association was only found between IgG aβ2-GPI and arterial thrombosis ($p=0.009$; **Table 3**). Elevated IgG aβ2-GPI levels were associated with venous thrombosis ($p=0.005$), and thrombocytopenia ($p=0.003$). More important, we found a close correlation between the aGPI titers and antinuclear antibody titers ($p=0.05$; **Figure 2**).

Discussion. The anti-human aβ2-GPI antibody used for characterization of the purification product by RID and ELISA showed no reaction in the RID, but reacted with the purification product in an ELISA in a manner that was consistent with a 2-sites binding. Therefore, it was proven by SDS-PAGE and ELISA that the purification product contained β2-GPI.[26] Phospholipids contamination of β2-GPI preparation was ruled out as use of a delipidated fraction of β2-GPI along with the naïve β2-GPI preparation for ELISA testing of the same serum samples, did not show any significant differences in OD readings between wells coated with naïve or delipidated β2-GPI. We could therefore conclude that there was no phospholipids present in our β2-GPI preparation. We have also found that estimation of non-specific binding (NSB) using non-antigen coated microtiter plate wells is absolutely necessary in the IgG aβ2-GPI ELISA, since many patients who had high OD, also had high NSB. As a result, failure to determine NSB increases the number of false positive samples, and reduces the specificity of the assay. It is of interest that all "false positive" patients were SLE patients and none of them suffered from primary antiphospholipid syndrome (PAPS). This can be explained by the fact that some SLE patients have autoantibodies in their sera, which could in some way bind to the uncoated wells and accounted for the increased NSB.[20-24] As this ELISA was developed without reference to an existing protocol, more parameters had to be varied to find the optimum conditions. The choice of microtiter plate was a crucial factor in the development of the aβ2-GPI ELISA. Binding properties of the 3 plates tested were quite different. Variation of the concentration of the coating antigen had practically no influence on the measurement of antibodies of the IgG isotype. The choice of blocking agent was another crucial factor. The BSA provided the best blocking effect, over a wide range of concentrations. The erratic behavior of gelatin may be explained by solidification at higher concentrations, giving a misleadingly high OD. The concentration of conjugate showed a linear relationship with the OD measured. Intra- and inter-assay precision of the assay were acceptable.

For reporting the results, we used aβ2-GPI units, assigning an arbitrary value of 100 units to a positive sample (calibrator), which was subsequently used in serial double dilutions to produce a standard curve. This way of reporting antibody levels measured by ELISA is considered the best as it gives low inter-assay variation, good reproducibility, and the potential for the use of an international unit of measurement. We found increased levels of IgG aβ2-GPI in approximately 90% of SLE patients. The concentration of IgG aβ2-GPI in SLE

patients is low in comparison with that reported in most previous studies, where the percentage of positives for IgG aβ2-GPI varies from 10.1%[27] to 86.6%[28] Many factors may be responsible for these discrepancies, especially as the patient population used was not the same in all studies. The largest study so far was carried out in Japan, with 308 SLE patients, and reported the lowest prevalence (10.1%) for raised IgG aβ2-GPI.[27] The number of patients receiving corticosteroid therapy at the time of each study was not reported, although it could decrease the number of patients testing positive for some autoantibodies.[27] Differences in the assay system and the definition of the cut-off point for positivity could account for the discrepancy in the result reported in different studies. The IgG aβ2-GPI ELISA has not as yet been standardised, and consequently there is wide interlaboratory variation in reported results. To ensure that the normal range was defined, and representative for the normal subjects, we used 50 normal blood donors. Multiple mechanisms have been proposed for thrombosis association, which might involve inhibition of the protein C pathway, inhibition of antithrombin III activity and enhanced endothelial cell procoagulant activity.[29] In vitro anticoagulant properties of IgG aβ2 are caused mainly by interference to binding of prothrombinase complex onto phospholipids surfaces.[6] It is also possible that in vivo IgG aβ2 could cause inhibition of phospholipids dependent reactions of the protein C pathway, a natural anticoagulant pathway, could enhance factor Xa generation by platelets, alternatively enhance platelet activation.[30]

In conclusion, the estimation of IgG aβ2 levels appears to offer a clear advantage, and may be used as a screening test in every day clinical practice. Autoimmune diseases are both sufficiently interesting and common to attract major research interest. The production of antibodies against autoantigens is one key feature of autoimmunity, which can easily be studied in humans. Fortunately and more important, the development of a validated assay for measurement of IgG aβ2 may provide an important tool for other researchers to gain further insight into the pathophysiology of autoimmunity. Lastly, animal studies may use this system to provide greater experimental freedom.

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