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A novel ELISA system for simultaneous detection of six subclasses of anti-phospholipid antibodies for prediction of thrombotic complications among SLE patients

Junzo Nojima *, Yukari Motoki, Natsumi Aoki, Hidehiro Tsuneoka, Kiyoshi Ichihara

Department of Laboratory Science, Faculty of Health Science, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755–8505, Japan

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ABSTRACT

Background: Anti-phospholipid antibodies (aPLs) are frequently associated with arterial and/or venous thromboembolic complications and recurrent fetal loss in patients with systemic lupus erythematosus (SLE). We recently reported that the clinical picture of SLE apparently depends on subclasses of aPLs in the patient's sera, but the contribution of each subclass remains uncertain.
Methods: We newly developed an ELISA system for simultaneous detection of six specific categories of aPLs: anti-cardiolipin (aCL), anti-β_2-glycoprotein I (aβ_2GPI), anti-cardiolipin/β_2-glycoprotein I (aCL/β_2GPI), anti-phosphatidylserine (aPS), anti-prothrombin (aPT), and anti-phosphatidylserine/prothrombin (aPS/PT). They were measured in 331 patients with SLE including 63 patients with arterial thromboembolic complications, 64 with venous thromboembolic complications, and 43 with recurrent fetal loss. Lupus anticoagulant (LA) activity in their plasma was measured according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies.
Results: Multivariate logistic analysis revealed that the concentration of aPS/PT was most closely associated with arterial thrombosis. In contrast, the concentration of aβ_2GPI was most closely related to venous thrombosis. Furthermore, both aCL/β_2GPI and aPS/PT were independently associated with episodes of recurrent fetal loss. Regarding the relation between APLs and LA activity, aPS/PT, followed by aβ_2GPI and aPT, showed the closest association with the presence of LA activity.
Conclusions: Anti-phospholipid syndrome in patients with SLE can be classified by antigenic specificities of their aPLs as to their susceptibility to arterial and/or venous thromboembolic complications or obstetric complications.

Introduction

Anti-phospholipid antibodies (aPLs) are a heterogeneous group of autoantibodies that appear in a variety of autoimmune diseases, particularly systemic lupus erythematosus (SLE) [1–3]. The presence of aPLs is associated with clinical events such as arterial and/or venous thrombosis and recurrent fetal loss [4,5]. Anti-phospholipid syndrome (APS) is currently diagnosed by both clinical findings (recurrent arterial and/or venous thrombosis and obstetric complications) and laboratory evidence of persistent aPLs [6,7]. At present, the most common and best characterized aPLs are anti-cardiolipin (aCL) and anti-β_2_glycoprotein I (aβ_2GPI), and lupus anticoagulant (LA) activity [5,6]. ACL and aβ_2GPI are measured by enzyme-linked immunosorbent assay (ELISA) [4,8]. LA activity is currently detected as the inhibitory effect of aPLs on certain in vitro phospholipid-dependent coagulation reactions [7].

A number of clinical studies have established that aPLs are present in approximately 40% of patients with SLE and that the presence of aPLs apparently constitutes a risk factor for arterial and/or venous thrombosis [3,9,10]. Moreover, recent reports indicated that the presence of LA activity detected by the phospholipid-dependent coagulation assay is closely linked with thrombotic events in patients with SLE [11,12]. However, the detection of LA activity involves a series of cumbersome procedures requiring careful quality control. According to the guidelines proposed by the Scientific and Standardization Committee on LA and phospholipid-dependent antibodies of the International Society of Thrombosis and Haemostasis [7,13], the laboratory diagnosis of LA should be done on plasma prepared by double centrifugation and

Abbreviations: aPLs, anti-phospholipid antibodies; SLE, systemic lupus erythematosus; APS, anti-phospholipid syndrome; aCL, anti-cardiolipin; aβ_2GPI, anti-β_2-glycoprotein I; LA, lupus anticoagulant; ELISA, enzyme-linked immunosorbent assay; aCL/β_2GPI, anti-cardiolipin/β_2-glycoprotein I; aPS, anti-phosphatidylserine; aPT, anti-prothrombin; aPS/PT, anti-phosphatidylserine/prothrombin; TBS, Tris-buffered saline; BSA, bovine serum albumin; TMB, tetramethylbenzidine; aOR, adjusted odds ratio; CI, confidence interval; aAUC, adjusted area under curve; ROC, receiver operating characteristic; mAU, milliabsorbance units.

* Corresponding author at: Department of Laboratory Science, Division of Health Science, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755–8505, Japan. Tel./fax: +81 836 22 2824.
E-mail address: nojima-j@yamaguchi-u.ac.jp (J. Nojima).

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should follow a four-step procedure respecting the following principles: 1) prolongation of a phospholipid-dependent coagulation assay, 2) evidence of inhibition demonstrated by mixing tests, 3) evidence of phospholipid-dependence demonstrated by confirmation tests, and 4) lack of specific inhibition of any one coagulation factor. Despite internationally accepted guidelines and many efforts to standardize procedures for LA assays, the laboratory diagnosis of LA remains to be harmonized.

Recent reports suggest that ELISAs specific for subclasses of aPLs may be of help to confirm the presence of LA activity [14–16]. It is ideal if a highly specific ELISA can replace the cumbersome assay for LA activity and can also predict clinical complications of each patient by allowing differential detection of aPLs. Therefore, we sought to develop a novel ELISA system for simultaneous detection of six aPL subclasses: aCL, aβ_2GPI, anti-cardiolipin/β_2-glycoprotein I (aCL/β_2GPI), anti-phosphatidylserine (aPS), anti-prothrombin (aPT), and anti-phosphatidylserine/prothrombin (aPS/PT). We applied this system to the differential detection of these aPLs in 331 patients with SLE including those with arterial thrombosis, venous thrombosis, and recurrent fetal loss.

Materials and methods

Study cohort

We studied plasma samples from 331 patients (311 females, 20 males; aged 8–82 years, mean 44.7 years) with SLE. Diagnosis of SLE was made according to the revised criteria of the American College of Rheumatology Criteria for Classification of Systemic Lupus Erythematosus. All the 331 patients with well-defined SLE have been clinically followed regularly for at least 10 years. All the thromboembolic complications occurred either at the onset or during the follow-up. Clinical history revealed that thromboembolic complications were observable in 127 of the 331 SLE patients: arterial thrombosis in 63 patients and venous thrombosis in 64 patients. All incidences of thrombosis had been documented by venography, arteriography, angiography, Doppler ultrasound, and/or computed tomography scan. We defined fetal loss, seen in 43 patients, as abortion, stillbirth, or dead fetus more than twice during our clinical observation. For the control group, we studied plasma samples from 200 normal healthy volunteers (174 women, 26 men; 22–60 years of age; mean, 42.5 years). None of these subjects had any history of thrombosis, and there were no abnormalities in their blood examinations (blood cell counts, coagulation tests, liver function tests, and examinations for autoimmunity). Blood samples were taken into vacuum tubes (5.0-mL total volume, SEKISUI, Japan) containing 0.5 mL of 3.13% trisodium citrate ($Na_3C_6H_5O_7\ 2H_2O$), and platelet-poor plasma was prepared by double centrifugation at 2800 $\times g$ for 15 min at 18 °C. The plasma samples were then frozen at −80 °C until batch assays could be performed. Since the LA assay requires the use of plasma samples, we employed plasma samples as a specimen for the ELISAs as well. However, we confirmed that serum and plasma gave identical results by the ELISAs. Informed consent was obtained from all patients and control subjects.

Detection of LA activity

Using an automated coagulation analyzer KC4 Delta™ (Tcoag Ireland Limited, Ireland), two clotting tests, activated partial thromboplastin time (APTT) and dilute Russell's viper venom time (dRVVT) test, were performed for LA activity determination according to the guidelines recommended by the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibodies [7]. For APTT, a sensitive reagent with low phospholipid concentration (PTT-LA, Diagnostica Stago) was used for screening test and mixing test. A mixing test (plasma samples:normal pooled plasma concentrations of 9:1, 4:1, 1:1, 1:4, and 1:9) was used to confirm the presence of LA activity. The dRVVT test was used to screen and confirm the presence of LA activity, using a LA-screen and LA-confirm reagents (Gradipore Ltd). The Staclot LA test (Diagnostica Stago) was also used to confirm the presence of LA activity.

Detection of aCL AND aCL/β_2GPI

The concentrations of aCL and aCL/β_2GPI were measured by a specific ELISA system. Polystyrene ELISA plates (Nunc-Immunoplate, Poly-Sorp; Kamstrup, Roskilde, Denmark) were coated with 30 μL (50 mg/L in ethanol) of cardiolipin (Sigma, St. Louis, MO) and dried for 3 h at room temperature. To avoid nonspecific binding of proteins, the wells were incubated 120 min at room temperature with 200 μL of Tris-buffered saline (TBS; 50 mmol/L Tris–HCl, 0.1 mol/L NaCl; pH 7.4) containing 10 g/L bovine serum albumin (BSA; Sigma). After each well had been washed 4 times with TBS containing 5 g/L BSA and 0.5 mL/L Tween 20, the wells were then incubated with 50 μl of TBS containing 5 g/L BSA (aCL wells) or 50 μl (20 mg/L in TBS containing 5 g/L BSA) of human β_2-GPI (SCIPAC, Sittingbourne, UK) (aCL/β_2GPI wells). After 30 min incubation at room temperature, 50 μL of plasma sample (diluted 101-fold with TBS containing 5 g/L BSA) was added to each well and incubated for 60 min at room temperature. Thereafter, the wells were washed with TBS containing 5 g/L BSA and 0.5 mL/L Tween 20 and then incubated with 100 μL of affinity-purified peroxidase-conjugated goat anti-human IgG (γ-chain specific) $F(ab')^2$ fragment (Sigma) for 60 min at room temperature. The wells were then washed 4 times with TBS containing BSA and Tween 20, and 100 μL of tetramethylbenzidine (TMB) solution (Moss Inc., Pasadena, MD) was added to each well. After 30 min incubation, the reaction was terminated by addition of 100 μL of 1 N HCl, and the absorbance was measured at 450 nm.

Detection of aPS AND aPS/PT

The concentrations of aPS and aPS/PT were measured by a specific ELISA system [8,17]. Polystyrene ELISA plates (Nunc-Immunoplate, Poly-Sorp; Kamstrup) were coated with 30 μL (50 mg/L in methanol) of phosphatidylserine (Sigma) and dried for 3 h at room temperature. To avoid nonspecific binding of proteins, the wells were incubated 120 min at room temperature with 200 μL of TBS containing 10 g/L BSA and 5 mmol/L $CaCl_2$. After each well had been washed 4 times with TBS containing 5 g/L BSA, 5 mmol/L $CaCl_2$, and 0.5 mL/L Tween 20, the wells were then incubated with 50 μl of TBS containing 5 g/L BSA (aPS wells) or 50 μl (20 mg/L in TBS containing 5 g/L BSA and 5 mmol/L $CaCl_2$) of human prothrombin (Enzyme Research Laboratories Inc., South Bend, IN) (aPS/PT wells). After 30 min incubation at room temperature, 50 μL of plasma sample (diluted 101-fold with TBS containing 5 g/L BSA and 5 mmol/L $CaCl_2$) was added to each well and incubated for 60 min at room temperature. The wells were then washed with TBS containing BSA, $CaCl_2$ and Tween 20 and incubated with affinity-purified horseradish peroxidase-conjugated goat anti-human IgG $F(ab')^2$ (γ-chain specific) fragment at room temperature for 60 min. Color reaction was developed with TMB solution (Moss, Inc.), and the absorbance was measured at 450 nm.

Detection of aβ_2GPI AND aPT

The concentrations of aβ_2GPI and aPT were measured by a specific ELISA system. γ-Irradiated polystyrene plates (Nunc-Immunoplate, Maxi-Sorp; Kamstrup) were coated overnight at 4 °C with 50 mL per well of β_2GPI (aβ_2GPI wells) or PT (aPT wells), each suspended at a concentration of 5 mg/L in TBS containing 5 g/L BSA. To avoid nonspecific binding of proteins, the wells were incubated 120 min at room temperature with 200 μL of TBS containing 10 g/L BSA. After each well had been washed 4 times with TBS containing 5 g/L BSA and 0.5 mL/L Tween 20, 50 μL of plasma sample (diluted 101-fold with TBS containing 5 g/L BSA) was added to each well. After 60 min of incubation at room temperature, the wells were washed with TBS-Tween. Horseradish peroxidase-