Significance of antiprothrombin antibodies in patients with systemic lupus erythematosus: clinical evaluation of the antiprothrombin assay and the antiphosphatidylserine/prothrombin assay, and comparison with other antiphospholipid antibody assays

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Introduction

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Methods currently used for detecting aPL can be divided to two categories: lupus anticoagulant (LAC) tests and EIA. Enzyme immunoassays are frequently used for detection of aPL, due to their relative simplicity and reliability. A standardized aCL EIA, described by Harris et al., is widely used for detection of aPL and the diagnosis of APS. However, during the last decade it has become evident that anticardiolipin antibodies are in fact autoantibodies against phospholipid binding proteins. The first such protein described is \(\beta_2\)-glycoprotein I (\(\beta_2\)GPI). It is suggested that \(\beta_2\)GPI undergo a conformational change upon binding to negatively charged phospholipids, which in turn causes exposure of neoepitopes that are detected by aCL. Alternatively, the density of \(\beta_2\)GPI may be important for recognition by aCL, and appropriate \(\beta_2\)GPI density is achieved by binding to negatively charged phospholipids. Enzyme immunoassay for detection of \(\beta_2\)GPI dependent aCL is reported to be of value in clinical practice and is included in the proposed Sapporo criteria for classification of APS. Enzyme immunoassays using \(\beta_2\)GPI directly coated on oxidized plates (high binding plates) are also reported to be useful.

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Original Article

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and prothrombin. Thereafter, a number of studies with regard to the relationship between thrombotic events and presence of antiprothrombin antibodies as measured by EIA have been reported, with conflicting conclusions.37 Interestingly, two methods for antiprothrombin antibody detection have been suggested, similar to the case for anti-β2GPI detection. One is an assay that utilizes prothrombin coated directly onto high binding plates (in this paper referred to as aPT-A), and the other is an assay that detects antibodies bound to prothrombin on phosphatidylserine-coated plates (in this paper referred to as aPS/PT). The large differences in the results among the reports that studied the relevance of antiprothrombin EIA may partly be due to the methods of the EIA employed in individual studies. However, very few studies have directly compared the aPT-A and the aPS/PT assays.18,19

In this study, we aimed to compare these two antiprothrombin EIA, along with other aPL EIA (IgG and IgM aCL, IgG â2GPI/CL) and LAC, for their values in assessing the risk of thrombosis in patients with systemic lupus erythematosus (SLE). In particular, for all EIA, we measured the values of each antibody in our own healthy controls and have set the cutoff points anew to enable comparison of these assays from a same viewpoint.

Material and methods

Subjects and samples

Serum and plasma samples were taken from 139 SLE patients followed at University of Tsukuba Hospital, Tsukuba, Ibaraki, Japan. All SLE patients fulfilled the 1987 ACR criteria20,21 for the classification of SLE. Among these patients, 16 had one or more documented episodes of thrombosis (11 with arterial thromboses, 8 with venous thromboses, 3 with both arterial and venous thromboses). Mean follow-up period at the time of blood sampling was 9.95 ± 8.23 (mean ± SD) years; 9.00 ± 9.49 years for patients with history of thromboses, and 10.07 ± 8.09 years for patients without history of thromboses. Warfarin was started in 11 patients after diagnosis of thrombosis, and in a mean follow-up of 2.64 ± 2.87 years, no additional thrombotic episodes were noticed. Samples from 148 healthy volunteers, with no apparent history of thrombosis or autoimmune diseases, served as controls. Written informed consent was obtained from all subjects of this study, and the study was approved by the ethics committee of the University of Tsukuba.

Antiphospholipid antibody assays

Antiprothrombin antibodies measurement using high binding plates

Nunc Maxisorp plates (Nalge Nunc, Rochester, NY) were used. One hundred microliters per well (µl/well) of human prothrombin (Haematologica Technologies, Essex Junction, VT, USA) in carbonate buffer, pH 9.6, at a concentration of 10µg/ml was coated onto wells at room temperature overnight. After blocking by 1% bovine serum albumin (BSA) in Tris-buffered saline including 5mM calcium (TBS), 100µl/well of sample sera diluted 1:200 in TBS with 1% BSA and 0.5% Tween-20 (TBS/Tw) were added to the wells, and the plates were incubated for 2h at room temperature. After washing with TBS/Tw, alkaline phosphatase labeled goat anti-human IgG antibody (American Qualex, SanClemente, CA, USA) diluted 1:2500 in TBS/Tw were added to the wells, and the plates were incubated for 1h at room temperature. After further washing, substrate was added and the optical density was measured at 405nM by an autoreader. A standard curve was always generated using a standard serum, the antibody concentration in which designated as 100 units/ml.

Anti-phosphatidylserine/prothrombin antibodies

For the measurement of aPS/PT, a commercially available EIA kit (Medical and Biological Laboratories, Nagoya, Japan)22 was used. The kit detects aPS/PT in the presence of calcium and follows, in principle, the method described by Atsumi et al.18 The wells of the plates are coated with phosphatidylserine/human prothrombin by the supplier. Sample sera were diluted at 1:100 with the supplied dilution buffer, and 100µl of each sample were added to the wells. The plates were incubated for 1h at room temperature, and bound antibodies were detected by a peroxidase labeled goat anti-human IgG antibody.

Anticardiolipin antibodies

Anticardiolipin antibodies were measured according to the methods described by Harris et al.,3 using a Mesacup cardiolipin kit (Medical and Biological Laboratories) and a Mesacup cardiolipin IgM kit (Medical and Biological Laboratories).

Anticardiolipin/β2-glycoprotein I antibodies

Serum â2GPI/CL were measured using anticardiolipin/β2-glycoprotein I antibody enzyme-linked immunosorbent assay (ELISA) kit (Yamasa Shoyu, Choshi, Japan). The kit is supplied with plates coated with cardiolipin. The wells were first incubated with sample dilution buffer with or without β2GPI. Sample sera were diluted at 1:101 with the supplied dilution buffer, and 50µl /well of each sample was added to both the â2GPI added and non-added wells. Plates were incubated for 30min at room temperature. Bound antibodies were detected by a peroxidase labeled anti-human IgG antibody. Samples were considered positive for â2GPI/CL when the values obtained from the β2GPI added wells exceeded the cutoff point, and were above the values obtained from β2GPI non-added wells.

Determination of cutoff levels for enzyme immunoassays

Although the cutoff levels for commercially available EIA tests are determined by the suppliers, the definition of a
cutoff level differs among assays, and different control populations are used for the determination of each cutoff level in an assay. Therefore, to compare various assays from a same viewpoint, the cutoff levels for all EIA were determined anew. For all assays, the cutoff levels were set at mean + 2SD of our control samples.

**Lupus anticoagulant assay**

Lupus anticoagulant assay was performed using an LA test kit (Gradiapore, North Ryde, Australia), which is based on a simplified dilute Russel viper venom time described by Exner et al.\(^2\) Ratios equal to or larger than 1.3 were considered positive for LA.

Statistical analyses

Relationships between the levels of EIA were compared by Spearman’s rank correlation test. Differences in titer of any aPL measured by EIA between SLE patients with history of thromboses and patients without such history were compared by the Mann–Whitney U-test. Positivity for any aPL assay and having histories of thromboses were compared by Fisher’s exact test. \(P\) values equal to or less than 0.05 were considered as statistically significant.

**Results**

Cutoff levels of enzyme immunoassays

The cutoff levels of all five EIA performed in this study were determined anew from the same 148 sample sera. The cutoff levels for aPT-A, aPS/PT, \(\alpha\beta2\)-GPI/CL, IgG aCL, and IgM aCL assays were 17.95, 17.83, 0.57, 15.43, and 5.69, respectively. Values above these cutoff levels were considered positive for a given assay.

Titer of various antiphospholipid antibodies in SLE patients with or without history of thrombotic episodes

The levels of each EIA were compared between patients with history of thrombosis and those without thrombosis. In all assays, patients with history of thrombosis had significantly higher values compared to those without such history (Fig. 1). The differences observed between patients with or without thrombotic episodes seemed especially large in aPS/PT, although it is difficult to compare the assays in this way since the “unit” in each assay was defined independently among each other.

Results of antiprothrombin antibodies detected using high binding plates and those of antiphosphatidylserine/prothrombin antibodies are significantly correlated with each other.\(^3\) We compared the values of aPT-A and aPS/PT among sera from SLE patients. These values were significantly correlated with each other (\(\rho = 0.514, P < 0.0001\) by Spearman’s rank correlation), compared to relationships among other aPL such as between aPT-A and \(\alpha\beta2\)-GPI/CL (Fig. 2 and results not shown). However, some sera had high value for only one or the other of those assays.

Positivity of antiphosphatidylserine/prothrombin antibody and/or antiprothrombin antibody is correlated with having histories of thrombotic episodes

Positivity for aPS/PT was significantly related with having history of thrombosis (Tables 1 and 2). aPT-A positivity was also significantly related with history of thrombosis.

Positivity of \(\beta2\)-GPI-dependent aCL and/or lupus anticoagulant is significantly correlated with having histories of thrombotic events

When the cutoff level of \(\alpha\beta2\)-GPI/CL was set at 3.5 units as recommended by the supplier, 17 were positive, among whom 5 had history of thrombotic episodes (\(P = 0.0282\) by Fisher’s exact test). When the cutoff level was adjusted using data from our own healthy controls, a more significant relationship was observed (Tables 1 and 2). The OD values equivalent to 0.6 units were around 0.060—0.070 in the presence of \(\beta2\)-GPI, and around 0.015—0.040 in the absence of \(\beta2\)-GPI (not shown). Being positive for LAC was also significantly associated with history of thrombosis.
aCL, 8 had history of one or more thrombosis episodes, yielding a $P$ value of 0.0091 as calculated by Fisher’s exact test. No association between IgM aCL positivity and history of thrombosis was seen, regardless of whether the cutoff point was set at the values set by the manufacturer (1 of 7 positive patients with history of thrombosis, $P = 0.5837$ by Fisher’s exact test), or that set by ourselves (Table 1).

Table 1. Relationship between positivity of antiphospholipid assays and history of thrombosis

|                | Thrombosis | Total | Odds ratio | 95% CI       | $P$ value |
|----------------|------------|-------|------------|--------------|-----------|
|                | Yes        | No    |            |              |           |
| aPT-A          | Positive   | 8     | 27         | 35           | 3.556     | 1.221–10.355 | 0.0278 |
|                | Negative   | 8     | 96         | 104          |           |           |       |
| aPS/PT         | Positive   | 8     | 22         | 30           | 4.591     | 1.555–15.560 | 0.0072 |
|                | Negative   | 8     | 101        | 109          |           |           |       |
| aβ2GPI/CL      | Positive   | 10    | 23         | 33           | 7.246     | 2.391–21.966 | 0.0005 |
|                | Negative   | 6     | 100        | 106          |           |           |       |
| IgG aCL        | Positive   | 5     | 12         | 17           | 4.204     | 1.250–14.148 | 0.0282 |
|                | Negative   | 11    | 111        | 122          |           |           |       |
| IgM aCL        | Positive   | 2     | 9          | 11           | 1.809     | 0.354–9.232 | 0.6158 |
|                | Negative   | 14    | 114        | 128          |           |           |       |
| LAC            | Positive   | 7     | 9          | 16           | 9.852     | 2.972–32.657 | 0.0004 |
|                | Negative   | 9     | 114        | 123          |           |           |       |

aPT-A, antiprothrombin antibody measured using high binding plates; aPS/PT, antiphosphatidylserine/prothrombin antibody; aβ2GPI/CL, β2-glycoprotein I dependent anticardiolipin antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant

Relationships between conventional aCL assays, lupus anticoagulant assay, and history of thrombosis

The relationship between positivity of IgG aCL and history of thrombosis was statistically significant when the cutoff value was set at mean + 2SD of our control samples (Table 1). When the cutoff level was set at 10 units, originally set by the manufacturer, among the 31 patients positive for IgG aCL, 8 had history of one or more thrombosis episodes, yielding a $P$ value of 0.0091 as calculated by Fisher’s exact test. No association between IgM aCL positivity and history of thrombosis was seen, regardless of whether the cutoff point was set at the values set by the manufacturer (1 of 7 positive patients with history of thrombosis, $P = 0.5837$ by Fisher’s exact test), or that set by ourselves (Table 1).
Positivity of antiphospholipid antibodies among SLE patients with history of thrombosis

The 16 SLE patients with history of thrombosis were assessed for their aPL positivity (Table 3). Thirteen patients were positive for at least one of the antiphospholipid antibody assays performed. While a combination of IgG aCL and LAC enabled us to judge 8 patients as positive for aPL, a combination of aPS/PT and αβ2GPI/CL indicated 10 as positive for aPL.

Table 2. Sensitivity, specificity, and positive predictive values of antiphospholipid assays for history of thromboses in patients with systemic lupus erythematosus

| Assay       | Sensitivity | Specificity | Positive predictive value |
|-------------|-------------|-------------|---------------------------|
| aPT-A       | 0.500       | 0.775       | 0.229                     |
| aPS/PT      | 0.500       | 0.821       | 0.267                     |
| αβ2GPI/CL   | 0.625       | 0.813       | 0.303                     |
| IgG aCL     | 0.313       | 0.902       | 0.294                     |
| IgM aCL     | 0.125       | 0.927       | 0.182                     |
| LAC         | 0.438       | 0.927       | 0.438                     |

Table 3. Positivity of antiphospholipid assays in patients with history of thrombosis

| Patient | Thrombosis | aPT-A | aPS/PT | αβ2GPI/CL | IgG aCL | IgM aCL | LAC |
|---------|------------|-------|--------|-----------|---------|---------|-----|
| 1       | A          | −     | −      | −         | −       | −       | −   |
| 2       | A          | −     | −      | −         | −       | −       | −   |
| 3       | A          | +     | +      | +         | −       | −       | +   |
| 4       | V          | +     | −      | +         | −       | +       | −   |
| 5       | V          | −     | −      | −         | −       | −       | +   |
| 6       | A, V       | +     | +      | +         | −       | −       | +   |
| 7       | A          | +     | −      | −         | +       | +       | +   |
| 8       | A          | +     | +      | +         | −       | +       | +   |
| 9       | A          | +     | +      | +         | −       | −       | +   |
| 10      | A          | −     | −      | −         | −       | −       | −   |
| 11      | V          | +     | −      | −         | −       | −       | −   |
| 12      | V          | −     | +      | −         | +       | +       | +   |
| 13      | A, V       | +     | +      | −         | −       | +       | +   |
| 14      | A          | −     | −      | −         | −       | −       | −   |
| 15      | V          | +     | +      | +         | +       | −       | −   |
| 16      | A, V       | +     | +      | +         | −       | +       | +   |

A, with history of arterial thromboses; V, with history of venous thromboses; aPT-A, antiprothrombin antibody measured using high binding plates; aPS/PT, antiphosphatidylserine/prothrombin antibody; αβ2GPI/CL, β2-glycoprotein I-dependent anticardiolipin antibody; aCL, anticardiolipin antibody; LAC, lupus anticoagulant

Summary of thrombotic history: 1. Cerebral infarction (CI), occurring before diagnosis of systemic lupus erythematosus (SLE). No additional events with low-dose aspirin. 2. Left atrial thrombosis, found at the time of diagnosis of SLE. No recurrence with warfarin plus low-dose aspirin. 3. CI, occurring 21 years after diagnosis of SLE, while on low-dose aspirin. No further events after addition of warfarin. 4. Deep vein thrombosis (DVT) and pulmonary embolism (PE), occurring 3 years after diagnosis of SLE, while on low-dose aspirin. No further events after addition of warfarin. 5. PE found 2 years after diagnosis of SLE; while without any anticoagulation, Dipyridamole was added. Warfarin added 10 years after diagnosis of SLE to delay the progression of pulmonary hypertension. 6. Old lung infarction noticed 22 years after diagnosis of SLE, while under low-dose aspirin. No further episodes. 7. CI occurred 15 years before occurrence of SLE. 8. DVT occurred in the first year after diagnosis of SLE while under low-dose aspirin. Warfarin was added and no further episodes noticed. 9. PE, found 17 years after diagnosis of SLE while drug free. No episodes after addition of warfarin. 10. CI, occurring before diagnosis of SLE. 11. DVT, occurring 4 years after diagnosis of SLE. No recurrence after addition of warfarin. 12. DVT, found at the time of diagnosis of SLE. No recurrence after addition of warfarin and low-dose aspirin. 13. DVT, PE, both found at the time of diagnosis of SLE. No recurrence after addition of warfarin and low-dose aspirin. 14. Myocardial infarction occurring 24 years after diagnosis of SLE. CI found by further examination. No recurrence after low-dose aspirin and ticlopidine hydrochloride. 15. DVT, occurring 5 years after diagnosis of SLE while under no anticoagulation. No recurrence after addition of warfarin and low-dose aspirin. 16. CI, PE, both found at the time of diagnosis of SLE. No recurrence after addition of warfarin
8 and 0.0337. Other assays did not yield significant $P$ values. However, the number of patients with history of recurrent fetal losses was too small to allow us to draw a reliable conclusion.

**Discussion**

Previous studies suggested that aPT-A and aPS/PT assays detect significantly different populations of autoantibodies and the results of the two assays may not correlate with each other. We applied the two assays to the same samples consisting of SLE patients and healthy individuals to assess the differences between the two assays. Results show that both assays, aPS/PT in particular, are of potential value in assessing the risk of thrombosis in SLE patients.

Very recently, Bertolaccini et al. also compared the results of aPT-A and aPS/PT in their cohort of 212 SLE patients. In their study they also found a significant relationship between the results of aPT-A and aPT/PS. Similar to our study, they also noted the presence of a number of patients with discrepant results. Thus, along with the results of our study, it is conceivable that aPT-A and aPT/PS recognize an overlapping, but not identical, population of autoantibodies. In their study, Bertolaccini et al. found a slightly stronger relationship between positivity of IgG aPS/PS and a history of thromboses than IgG aPT-A positivity and a history of thromboses. However, they have not documented how the aPS/PT-positive and aPT-A-positive patients overlap, when the presence of thrombotic history of thrombosis was put into consideration. In our study, between 8 aPT-A-positive SLE patients with a history of thrombosis and 8 aPS/PT-positive patients with a history of thrombosis, only 5 overlapped. These results could imply that performing both aPT-A and aPS/PT assays, if possible, or developing a new method that would enable detection of both populations of autoantibodies would be desirable for more sensitive detection of clinically relevant antiprothrombin antibodies. The reason why aPT-A and aPS/PT assays recognize overlapping, but not identical, populations of autoantibodies is not clear, but a most reasonable explanation would be that the nature of conformational changes introduced to prothrombin is different between when prothrombin is bound to high binding plates and when it is bound to phosphatidylinerine bound plates. It is possible that the conformational changes introduced when bound to phosphatidylinerine-coated plates is more similar to the changes introduced in vivo, and this may account for the higher clinical relevance of aPS/PT assay than that of aPT-A assay observed in our study.

Assay for aβ2GPI/CL has become increasingly popular in the clinical management of SLE in Japan. The assay has been approved by the Ministry of Science Health and Welfare of Japan as a tool for diagnosis of APS. As seen in Table 1, positivity for this antibody was strongly correlated with histories of thrombosis, suggesting its value in a clinical setting. However, the cutoff value of aβ2GPI/CL in this study was set at 0.57 units, different from 3.5 units recommended by the manufacturer of the assay kit. In addition, the OD values at 0.6 units were around 0.060 in the assay we used. Thus, while setting the cutoff value at this level does give us a better $P$ value, implying the validity of the assay, refinement of the assay would be necessary to more accurately measure low-titer aβ2GPI/CL. By standardizing the methodology and appropriately setting the cutoff levels, aβ2GPI/CL could become a more widely used measure for the diagnosis of APS. Efforts to standardize this assay are necessary. Previously, we have suggested that aβ2GPI/CL assays may be suitable to be included in the 1987 ACR criteria, which currently includes only LAC and conventional aCL assays as means of aPL detection. Results presented in this study imply that aβ2GPI/CL EIA has similar or superior value for the detection of aPL as IgG aCL. We believe that inclusion of aβ2GPI/CL in the ACR criteria for SLE may be beneficial.

Currently, the Sapporo criteria, proposed in 1999, are widely used for the diagnosis of APS. In the Sapporo criteria, for the detection of aPL, aCL, aβ2GPI/aCL, and LAC assays are recommended. However, studies reported thereafter have suggested the values of antiprothrombin assays in the diagnosis of APS. We wished to determine the values of aPT-A and aPS/PT in a clinical setting and in particular, wanted to determine whether routine measurement of these antibodies would aid in the diagnosis of APS. For this purpose, we decided to set the cutoff level of each EIA anew, to evaluate each assay from the same viewpoint.

Among our 16 SLE patients with history of thrombosis, 8 were judged positive for aPL by the combination of IgG aCL and LAC. Addition of aβ2GPI/aCL raised the number of patients positive for aPL to 12, while addition of either aPT-A or aPS/PT raised the number to 11. A combination of aβ2GPI/aCL and aPS/PT judged 10 patients as positive for aPL, and a combination of aPT-A, aPS/PT, and aβ2GPI/aCL judged 12 as aPL-positive (Table 3). In our SLE patients, among the patients with history of thrombosis, all but one of those positive for LAC were positive for at least either aPS/PT or aβ2GPI/aCL. These results are quite reasonable since a large part, if not the majority, of LAC activity is β2GPI or prothrombin dependent. Refinement of these assays may show that these assays should have a position in the diagnostic criteria of APS, and that they have the potential to substitute LAC assays in the future. Although the clinical significance of LAC assays is well established, carefully collected and preserved plasma needs to be used, the methods are tedious and are still not completely standardized.

By directly comparing aPS/PT and aPT-A assays along with other aPL assays, using the same samples and the same criteria to set cutoff levels, our current study indicated that aPS/PT and aβ2GPI/aCL assays have more clinical relevance than the aPT-A assay. However, aPT-A assay seemed to be detecting a spectrum of autoantibodies not detected by aPS/PT, and we believe that it is premature to dismiss the assay as clinically meaningless.

In conclusion, we have shown the heterogeneity of autoantibodies detected by aPT-A or aPS/PT assays, but have also shown that these EIAs may be valuable in the
diagnosis of APS. Although interlaboratory studies and standardization of the assays are necessary, we believe that these assays may have the potential to be included in the future diagnostic criteria for APS and SLE.

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