ORIGINAL ARTICLE

Study on Anti-phospholipid Antibodies in Chronic Idiopathic Thrombocytopenic Purpura

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Abstract

We have developed a new solid phase enzyme immunoassay (EIA) for detection of anti-phospholipid antibodies in sera and platelet eluates from patients with idiopathic thrombocytopenic purpura (ITP). Antibodies against cardiolipin (CAR), phosphatidylinositol (PI) and phosphatidylserine (PS), not against phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and sphingomyelin (SM) were frequently found in the ITP patients' sera. Among 69 ITP patients examined, IgG class antibodies against CAR, PI and PS were detected in 12, 10 and 9 patients and IgM class antibodies in 16, 18 and 13 patients, respectively. When platelet eluates were analysed instead of sera, anti-phospholipid antibodies in IgG class were detected in 14 cases out of 58 patients (anti-CAR 4, anti-PI 13 and anti-PS 5), while the antibodies of IgM class were found in only one case. Although severe thrombocytopenia was found in one ITP patient with the exceptionally high titer of anti-phospholipid antibody, there were no clear correlation between the platelet counts and the titers of anti-phospholipid antibodies in platelet eluates. Thus, it is suggested that anti-phospholipid antibodies do not play a major role in pathogenesis of thrombocytopenia in spite of such a high incidence. There were no distinct differences in clinical and laboratory features between cases with and without anti-phospholipid antibodies. However, it was of great interest that 2 ITP patients with high titers of antibodies against all three phospholipids (CAR, PI and PS) presented unique complications such as deep vein thrombosis and lupus anticoagulant, a risk factor for thrombosis.

Key words: immune thrombocytopenia, platelet antigens, phospholipids, platelet eluates, thrombosis
Anti-phospholipid Antibodies in ITP

Introduction

ITP has been defined to be an autoimmune disease characterized by the destruction of platelets due to autoantibodies reactive with the platelet membranes, but the autoantibodies are not well characterized yet.1-4 With the development of immunological methodology for detection of antibodies on platelet membrane, increased levels of platelet-associated IgG (PAIgG) can be demonstrated on platelets from almost all patients with ITP. The autoantibodies reactive with platelet glycoprotein Ib (GPIb), a receptor of von Willebrand factor playing an important role in platelet adhesion to vessel wall, or glycoprotein IIb/IIIa (GPIIb/IIIa), a receptor of fibrinogen indispensable for platelet aggregation, were first recognized by Woods et al. in sera of patients with chronic ITP.5,6 The incidence of autoantibodies reactive with these major glycoproteins appears to be low, although some patients possessed both anti-GPIb and GPIIb/IIIa antibodies.7 It has been also demonstrated that radiolabelled splenic IgG from chronic ITP patients bound to multiple platelet proteins.8 These findings suggest that there exist various platelet autoantigens in ITP.

Recently, the antibodies against CAR were also shown to be present in sera from patients with chronic ITP—14 cases in IgG class antibody and 27 cases in IgM class out of 96 cases.9,10 Although Harris et al. suggested the causal relationship between anti-CAR antibodies and thrombocytopenia, the solid evidence has been lacking.

In this paper, we have also shown that anti-phospholipid antibodies against CAR, PI and PS, either IgG or IgM class, were frequently demonstrated in sera from patients with chronic ITP by EIA. To clarify the clinical significance of these antibodies, we have tried to answer the following questions: (A) Are these anti-phospholipid antibodies in sera associated with platelets? (B) Do they play any significant roles in pathogenesis of thrombocytopenia? (C) Are there any differences in clinical features between ITP cases with and without these antibodies?

Patients

ITP was diagnosed by the criteria of ITP study group of the Ministry of Health and Welfare, Japan, on the basis of thrombocytopenia, increased or normal numbers of megakaryocytes and no other demonstrable causes of thrombocytopenia.11 Sixty-nine patients were examined, 52 females and 17 males from 18 to 75 years old. (The mean age was 43.3 years old.) The platelet counts ranged from 4,000/μl to 91,000/μl (the mean value was 32,100/μl), when the patients visited the hospital for the first time. The onset was from 12 to 54 years old (the mean age was 34.2 years old). Splenectomy was performed in 37 patients because of inadequate response to prednisolone. Various autoantibodies in sera were measured. The anti-nuclear antibody (ANA) was found in 16 cases, the lupus erythematosus cell (LE cell) in 2, the rheumatoid factor (RF)
in 6, the thyroglobulin hemoagglutinin (TGHA) in 5, the microsome hemoagglutinin (MSHA) in 10, the Coombs' test in 5 and the biological false positive for syphilis (BFP) in 4, respectively.

Materials and Methods

(1) Sera and platelet eluates from patients with chronic ITP.

Sera were obtained from 69 patients with chronic ITP (17 males and 52 females) and 15 normal individuals, and were stored at −80°C until use. Platelet eluates were obtained from 58 patients or 15 normal individuals according to the method of McMillan et al.1 Washed platelet suspensions were prepared in phosphate buffered saline (PBS), pH 7.4, and the pH was lowered to 2.5 by adding 1N HCl to platelet suspensions. The supernatants were obtained by centrifugation at 3,000 r.p.m. for 15 minutes and were neutralized immediately.

(2) Assay of anti-phospholipid antibodies by EIA.

Seven kinds of phospholipids (CAR, PI, PS, PC, PE, PA and SM) were purchased from Sigma Chemical Co. USA.

Miscelles of each phospholipid were created in PBS, pH 7.4, by sonication for 60 minutes at the maximum output. Each well of polyvinylchloride plates was first coated with 50 µl of phospholipid suspension (1 mg/ml in 0.01 M PBS) by incubation overnight at 4°C. After inverting the wells containing the phospholipid suspension, 200µl of 10% of fetal calf serum (FCS) was added to each well and incubated for 60 minutes at 22°C to block any remaining protein-binding sites on the well surface. And the wells were washed in PBS containing 0.05% of Tween-20.

Assay of anti-phospholipid antibodies in sera or eluates by EIA was performed as follows. 100 µl of 1 : 100 dilution of sera or platelet eluates from 10⁷ platelets was added to each phospholipid-coated well and incubated for 120 minutes at 22°C. After five washes in PBS containing 0.05% of Tween-20, 50 µl of goat peroxidase-labelled anti-human IgG or IgM was added to each well and the plate was further incubated for 120 minutes. Finally, 100 µl of o-phenylenediamine in citrate phosphate buffer containing 0.03% hydrogen peroxide, pH 5, was applied. After incubation for 30 minutes in the dark, the optical absorbance was measured at 495 nanometers.12,13

The results were expressed as OD ratio according to the following formula:

$$\text{OD ratio} = \frac{\text{OD}_{495} \text{ of patients sera wells}}{\text{Mean OD}_{495} \text{ of normal sera wells}}$$

Results

(1) Anti-phospholipid antibodies in sera.

The titers of anti-phospholipid antibodies were expressed by OD ratio. OD ratio
Fig. 1 The titers of IgG class anti-phospholipid antibodies in sera from 69 patients with ITP.

Sera from patients or normal controls were incubated with each sonicated phospholipids—CAR, PI, PS, PC, PE, PA and SM—in microtiter wells. Then goat peroxidase-labelled anti-human IgG was added. Using o-phenylenediamine as substrate, the optical absorbance was measured at 495 nm. The OD ratio (OD of patients sera wells/mean OD of normal sera wells) of 3SD above the normal mean of 15 controls was shown by the dotted lines. By the closed circles, the titers of IgG class antibodies against CAR, PI and PS in patients' sera were expressed as OD ratio. The antibodies against the other phospholipids were not detected. The left column: the titers of anti-CAR antibodies, the middle: anti-PI antibodies and the right: anti-PS antibodies in sera from patients with ITP.

of 3SD above the normal mean of 15 controls was judged as positive. There were no elevation of OD ratio of patients' sera when PC, PE, PA and SM were used as antigens. On the other hand, anti-CAR, anti-PI and anti-PS antibodies were frequently demonstrated in sera from patients with chronic ITP. Out of 69 cases IgG class antibodies against CAR, PI, PS were detected in 12, 10 and 9 cases, respectively (Fig. 1). IgM class antibodies were more frequently demonstrated in sera. Anti-CAR in 16, anti-PI in 18 and anti-PS in 13 (Fig. 2). Titers of IgM class antibodies appeared to be higher than those of IgG class antibodies.
Fig. 2 The titers of IgM class anti-phospholipid antibodies in sera from 69 patients with ITP.

Sera from patients or normal controls were incubated with each sonicated phospholipids—CAR, PI, PS, PC, PE, PA and SM—in microtiter wells. Then goat peroxidase-labelled anti-human IgM was added. Using o-phenylenediamine as substrate, the optical absorbance measured at 495 nm. The OD ratio (OD of patients sera wells/mean OD of normal sera wells) of 3SD above the normal mean of 15 controls was shown by the dotted lines. By the closed circles, the titers of IgM class antibodies against CAR, PI and PS in patients’ sera were expressed as OD ratio. The antibodies against the other phospholipids were not detected. The left column: the titers of anti-CAR antibodies, the middle: anti-PI antibodies and the right: anti-PS antibodies in sera from patients with ITP.
Fig. 3 The incidence of IgG/IgM class anti-phospholipid antibodies in sera from 69 patients with ITP.

The OD ratio of 3SD above the normal mean of 15 controls was judged as positive. The number of positive values of IgG/IgM class anti-phospholipid antibodies was shown in each column. IgG class: the upper column, IgM class: the lower column.

The number of cases with IgG/IgM class anti-phospholipid antibodies was summarized in Fig. 3. It was clearly shown that IgM class antibodies against any phospholipid (CAR, PI or PS) were more frequently detected than IgG class antibodies. IgG class antibodies against all of CAR, PI and PS were found in only 3 cases, while IgM class antibodies were detected in 11 cases.

The relation between the platelet counts and the titers of anti-phospholipid antibodies was studied. The titers of either IgG class or IgM class antibodies against CAR, PI and PS did not correlate to the platelet counts (Figs. 4a and 4b).

(2) Anti-phospholipid antibodies in platelet eluates.

The elution of antibodies from platelets of patients with ITP and normal individuals was performed and anti-phospholipid antibodies were investigated in platelet eluates. In contrast to the results of sera, antibodies against CAR, PI and PS were less frequently found in platelet eluates. IgG class antibodies against CAR, PI and PS were found in 4, 13 and 5 cases, respectively (Fig. 5).

Fig. 6 summarized the number of cases with IgG/IgM class anti-phospholipid
Figs. 4a and 4b The relation between the platelet counts and the titers of IgG class (Fig. 4a) or IgM class (Fig. 4b) antibodies in sera from patients with ITP.

The horizontal dotted lines showed the OD ratio of 3SD above the normal mean of 15 controls. The left: anti-CAR antibodies, the middle: anti-PI antibodies and the right: anti-PS antibodies.

antibodies in platelet eluates from 58 patients of ITP. IgG class antibodies against all of three phospholipids, CAR, PI and PS, were detected in only 3 cases. IgM class antibodies, however, were recognized in only one case against all of CAR, PI and PS.

(3) The relation between the platelet counts and titers of anti-phospholipid antibodies in platelet eluates.

To clarify the significance of anti-phospholipid antibodies in pathogenesis of thrombocytopenia, the relation between the platelet counts and the titers of anti-phospholipid
Fig. 5 The titers of anti-phospholipid antibodies in eluates from platelets of 58 patients with ITP (IgG Class).

Eluates from patients' or normal washed platelets obtained by lowering pH to 2.5 were applied to each phospholipid coated well after neutralized and incubated in the same method as the sera. The titers of IgG class antibodies against CAR, PI and PS were expressed as the OD ratio. The horizontal dotted lines showed the OD ratio of 3SD above the normal mean of 15 controls. The left lane: the titers of anti-CAR antibodies, the middle: anti-PI antibodies and the right: anti-PS antibodies in eluates from the patients' platelets.

antibodies, IgG class, in platelet eluates was investigated. As shown in Fig. 7, most patients revealed OD ratio within normal limits irrespective of the platelet counts, which ranged from less than 10,000/μl to more than 200,000/μl. There were no clear relations between these two parameters. However, in case (A) who showed the highest titer of anti-phospholipid antibodies, the dramatic improvement of thrombocytopenia was accompanied by the decrease in the titer of the anti-phospholipid antibodies following prednisolone administration (Table 1).
Fig. 6 The incidence of IgG/IgM class anti-phospholipid antibodies in eluates from platelets of 58 patients with ITP.

The OD ratio of 3SD above the normal mean of 15 controls was judged as positive. The number of positive values of anti-phospholipid antibodies was shown in each column. IgG class: the upper column, IgM class: the lower column.

Table 1 The Effect of Prednisolone Administration on the Titers of IgG/IgM Class Anti-phospholipid Antibodies in Platelet Eluates and the Platelet Counts in Case (A)

<table>
<thead>
<tr>
<th>Titers of antibodies against</th>
<th>Before treatment</th>
<th>After PSL therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>CAR</td>
<td>5.3</td>
<td>1.9</td>
</tr>
<tr>
<td>PI</td>
<td>4.9</td>
<td>2.1</td>
</tr>
<tr>
<td>PS</td>
<td>3.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Platelet counts (×10⁴/µl) 18 142

The titers of anti-phospholipid antibodies were expressed by OD ratio.

The effect of splenectomy on the titers of anti-phospholipid antibodies and platelet counts was examined in four patients, Case (C), (D), (E) and (F) (Table 2). In all patients, the titers of anti-phospholipid antibodies in platelet eluates did not decrease after splenectomy in spite of marked improvement of thrombocytopenia.
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Fig. 7 The relation between the platelet counts and the titers of anti-phospholipid antibodies in eluates of platelets from patients with ITP.

The titers of anti-phospholipid antibodies were shown by OD ratio. The horizontal dotted lines showed OD ratio of 3SD above normal mean of 15 controls. The left: anti-CAR antibodies, the middle: anti-PI antibodies and the right: anti-PS antibodies.

Table 2 The Effect of Splenectomy on the Titers of Anti-phospholipid Antibodies (IgG Class) in Platelet Eluates and the Platelet Counts

<table>
<thead>
<tr>
<th>CASE</th>
<th>Titers of Antibodies against</th>
<th>Platelet Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAR</td>
<td>PI</td>
</tr>
<tr>
<td>(C)</td>
<td>before</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>2.0</td>
</tr>
<tr>
<td>(D)</td>
<td>before</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>1.6</td>
</tr>
<tr>
<td>(E)</td>
<td>before</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>1.3</td>
</tr>
<tr>
<td>(F)</td>
<td>before</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>after</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The titers of anti-phospholipid antibodies were expressed by OD ratio.

(4) Clinical features in patients with positive anti-phospholipid antibodies.

It is well known that some of ITP patients possessed the other autoantibodies such as ANA, LE cell, RF, TGHA, MSHA, Coombs' test and BFP. The relation between these autoantibodies and the anti-phospholipid antibodies in patients with ITP was investigated. In Table 3, the incidence of these coexistent autoantibodies in sera was recorded in patients with or without anti-phospholipid antibodies. There was no signifi-
Table 3 The Incidence of Coexistent Autoantibodies with Anti-phospholipid Antibodies in Sera from 69 Patients with ITP

<table>
<thead>
<tr>
<th></th>
<th>ANA</th>
<th>LE cell</th>
<th>RF</th>
<th>TGHA</th>
<th>MSHA</th>
<th>Coombs</th>
<th>BFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA(+)</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>APA(−)</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>1−0</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

ANA, LE cell, RF, TGHA, MSHA, Coombs' test and BFP were measured. The number of cases with these antibodies was listed in two groups which possessed anti-phospholipid antibodies [APA(+)] or not [APA(−)].

Table 4 Characters of Anti-phospholipid Antibodies in Sera from ITP Patients with BFP

<table>
<thead>
<tr>
<th>CASE</th>
<th>anti-phospholipid antibodies against</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C)</td>
<td>CAR (IgG, M), PI (IgG, M), PS (IgG)</td>
</tr>
<tr>
<td>(G)</td>
<td>CAR (IgM), PI (IgM), PS (IgM)</td>
</tr>
<tr>
<td>(H)</td>
<td>CAR (IgG), PS (IgG)</td>
</tr>
<tr>
<td>(I)</td>
<td>(−)</td>
</tr>
</tbody>
</table>

Case (C), (G), (H) and (I) revealed BFP. The antibodies against PC, PE, PA and SM were not detected in any case.

Significant correlation in the incidence of autoantibodies between these two groups of patients. Four cases showed BFP out of 69 cases. Three cases (case C, G and H) out of 4 cases with BFP possessed the anti-phospholipid antibodies against cardiolipin in IgG and/or IgM class in the assay for anti-phospholipid antibodies by solid phase EIA which was shown in Table 4. But one case (case I) with BFP did not show the anti-phospholipid antibodies. BFP was observed when the patient was first diagnosed as ITP in 1981, but soon became negative after prednisolone therapy in this case. The assay for anti-phospholipid antibodies was performed while treated by prednisolone.

It is important to note that patients with high titers of antibodies against all of CAR, PI and PS presented unique clinical features.

Case (A) with the highest titers of anti-phospholipid antibodies—the titers of anti-CAR 5.3, anti-PI 4.9, anti-PS 3.7 as mentioned already, was a 69-year-old female suffering from Takayasu's disease. Case (B) was a 56-year-old male with deep vein thrombosis from the left brachial vein to the subclavian vein, who revealed the second highest titers of anti-phospholipid antibodies—anti-CAR 3.0, anti-PI 3.0 and anti-PS 3.0. Case (C) was a 34-year-old male with lupus anticoagulant. His titers of anti-phospholipid antibodies against CAR, PI and PS were 2.0, 1.5 and 2.9, respectively (Table 5). The
Table 5 Titers of Anti-phospholipid Antibodies (IgG Class) in Platelet Eluates from ITP Patients

<table>
<thead>
<tr>
<th>Case</th>
<th>anti-CAR</th>
<th>anti-PI</th>
<th>anti-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>5.3</td>
<td>4.9</td>
<td>3.7</td>
</tr>
<tr>
<td>(B)</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>(C)</td>
<td>2.0</td>
<td>1.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

(A): a case with Takayasu's disease, which revealed the highest titers of antibodies against CAR, PI and PS
(B): a case with deep vein thrombosis
(C): a case with lupus anticoagulant

coexistence of these vascular or thrombotic disorders in patients with ITP, which has not been reported in Japanese literature until now, is worth while to be mentioned.

Discussion

Our findings that anti-CAR antibodies were frequently demonstrated in sera from patients with chronic ITP were fairly comparable with those observed by Harris et al. Harris et al. found 30 out of 96 patients with chronic ITP revealed anti-CAR antibodies in their sera, more frequently in IgM class (27 cases) than in IgG class (14 cases). Not only anti-CAR antibodies we have also demonstrated anti-PI, anti-PS antibodies in sera. These results were expected because Thiagarajan et al. showed that the lupus anticoagulant was anti-phospholipid antibodies against negatively charged phospholipids such as CAR, PI and PS. Although Harris et al. suggested the causal relationship between thrombocytopenia and anti-CAR antibodies, the definite evidences were lacking. They showed no evidence that anti-CAR antibodies could react with platelets. In addition, CAR is only a minor component of platelet phospholipids and is known to be present inside the platelets, not exposed to the outer surface of platelet membranes. PI and PS are also known to be lining the inner layer of platelet membrane and rarely exist on the outer surface in non-stimulated platelets. Therefore, in order to decide pathogenic roles of anti-phospholipid antibodies, it is important to investigate whether platelet eluates include anti-phospholipid antibodies or not. Unexpectedly, both IgG and IgM class antibody levels were less frequently elevated in platelet eluates as compared with in sera. Especially, IgM class antibodies were rarely demonstrated in platelet eluates. Only one patient (case A), who has been suffering from Takayasu's disease with ITP, has IgM class antibodies with extremely high titers of IgG class antibodies in platelet eluates. It is fascinating to speculate that the phospholipids of the affected vascular vessels might participate in producing the antibodies towards both vascular walls and platelets. In fact, severe thrombocytopenia (platelet count 18,000/μl) was successfully treated (platelet
count increased to 146,000/µl) with 30 mg of prednisolone, which also lowered the titers of anti-phospholipid antibodies to normal range. It is certain that a part of the anti-phospholipid antibodies present in sera could react with platelets. Therefore, anti-phospholipid antibodies were partly considered to be a subclass of platelet bound IgG or IgM.

IgG class antibodies, on the other hand, were present in platelet eluates from some patients with chronic ITP besides case (A), although there were no clear relation between the platelet counts and the titers of anti-phospholipid antibodies of IgG class. Therefore, it seems that anti-phospholipid antibodies may not mediate peripheral platelet destruction in most cases with ITP.

Our results differ from those obtained by Harris et al. The reason for this discrepancy is not clear at present time. Harris et al. showed the significant relation between anti-phospholipid antibodies in sera, not in platelet eluates, and thrombocytopenia, while we measured anti-phospholipid antibodies in both sera and platelet eluates and found no significant correlation.

Recent studies suggested that lupus anticoagulant, which was most likely anti-phospholipid antibodies against PS or PI, can be recognized as a risk factor of thrombosis. High levels of anti-phospholipid antibodies against all of CAR, PI and PS were noted in platelet eluates from three patients (case A, B and C). Case (B) developed deep vein thrombosis and case (C) had evidence of lupus anticoagulant. Deep vein thrombosis or lupus anticoagulant associated with ITP, a thrombotic disorder coexisting with a bleeding disease, has not been reported in Japanese literature. The author et al. reported both cases. Unusual coexistence of these two disorders may not be coincidentally associated with high titers of anti-phospholipid antibodies against all of CAR, PI and PS.

It is fascinating to speculate that presence of anti-phospholipid antibodies against CAR, PI and PS may select a population of patients with thrombotic disorders.

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References