

## Multidirectionality of Anti-Platelet Antibodies in Idiopathic Thrombocytopenic Purpura

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### ABSTRACT

Chronic idiopathic thrombocytopenic purpura (chronic ITP) is an autoimmune disorder characterized by bleeding tendency and the presence of auto-antibody to platelets. Quantitative analysis of the level of anti-platelet antibodies and platelet associated antibodies is now possible. Furthermore, recent immunological techniques have enabled us to detect target platelet antigens in chronic ITP.

In this study, (1) anti-platelet antibodies, contained in serum, were examined by an indirect platelet suspension immunofluorescence test (indirect PSIFT) for IgG+A+M, and platelet bindable IgG (PB-IgG) was measured quantitatively by enzyme linked immunosolvent assay (ELISA). Indirect PSIFT was positive in 4.2%, and PB-IgG was elevated in 12.5% of ITP patients. (2) Platelet associated antibodies were detected by direct PSIFT and platelet associated IgG (PA-IgG) were assayed by solid phase competitive ELISA. Direct PSIFT was positive in 34.1% and PA-IgG was elevated in 37.8% (3) IgG antibodies against platelet lipid compounds (cardiolipin and sulfatides) were also measured by ELISA. Anti-cardiolipin antibody was elevated in 56.3% and anti-sulfatides antibody was elevated in 31.3%. Anti-cardiolipin antibodies correlated well with anti-sulfatides antibody, and also correlated with PB-IgG. (4) Target platelet antigens were detected by immunoprecipitation and Western blotting. Immunoprecipitation showed multiple antigenic bindings in one out of 19 ITP patients with positive serum anti-platelet antibody. On Western blotting analysis, multiple antigenic binding sites were detected in six out of 48 patients.

The data in this study are consistent with the idea that auto-antibodies in ITP are not directed towards a single protein, but to multiple platelet antigens which are included in proteins and lipid compounds.

**Key words:** ITP, PA-IgG, Platelet antigen

Idiopathic thrombocytopenic purpura (ITP) has been known from the time of Werlhof's report more than 200 years ago. Following the demonstration of circulating anti-platelet factor in the plasma of chronic ITP patients by Harrington et al<sup>15)</sup> in 1951, dozens of different techniques for the detection of anti-platelet antibodies have been reported. Now, there is general agreement that ITP is an autoimmune disease well characterized by anti-platelet antibody<sup>21,22,29)</sup>. Platelet associated IgG (PA-IgG) was first quantitatively measured using the complement lysis inhibition assay in 1975 by Dixon et al<sup>11)</sup>. They demonstrated a strong inverse correlation between PA-IgG and platelet count in chronic ITP patients. Conversely, elevation of PA-IgG has been found in various thrombocytopenic disorders<sup>23)</sup>. Although elevation of PA-IgG is still important in the diagnosis of chronic ITP, it is now thought that elevation of PA-IgG is not specific for chronic ITP.

PA-IgG are thought to be located at the various

sites in platelet, for example, at platelet specific antigens or at platelet Fc receptor. Platelet glycoprotein (GP) IIb/IIIa was first estimated to be a target platelet antigen in chronic ITP<sup>26)</sup>. Another glycoprotein, GP Ib, was also reported to be a target antigen. Recently, platelet component lipids, cardiolipin and glycosphingolipids, were reported to be target antigens<sup>18,44)</sup>. However, no target antigen which is commonly present in ITP has been reported. The present study was designed to examine the significance of platelet antibodies by different methods, and to characterize the target antigen distribution of ITP.

### MATERIALS AND METHODS

#### 1) Materials

Random samples were collected from patients with chronic ITP in Hiroshima University Hospital from April 1985 to December 1988. The samples did not overlap within each examination. Diagnosis of chronic ITP was based on the diagnostic

criteria of the Idiopathic Hematopoietic Disorders Research Committee<sup>32)</sup>, supported by the Ministry of Health and Welfare of Japan. Data were statistically analyzed by personal computer PC-9801 (NEC, Tokyo) with statistical software (STAX, Nakayama Publish. Inc., Tokyo).

## 2) Platelet suspension immunofluorescence test (PSIFT)

Platelet associated antibodies and platelet bindable antibodies (IgG, A, and M) were examined employing a modification of the platelet suspension immunofluorescence test (PSIFT) of von dem Borne et al<sup>5)</sup>.

### Direct immunofluorescence test

Isolation of patient platelets: Patient blood was collected in a disposable plastic syringe containing 10% V/V of 3.8% sodium citrate. Platelet-rich plasma (PRP) was separated by centrifugation during 15 min at 150g, 22°C. Ten percent V/V of ACD-A solution (Terumo, Tokyo) was added to PRP. Then platelets were pelleted for 10 min at 1,400g. Platelets were washed 4 times with citrate buffer at pH 6.6 and were fixed for 5 min at room temperature by 1% paraformaldehyde (PFA) in 13 mM phosphate buffered saline at pH 7.2 containing 9 mM ethylene diamine tetra acetate (EDTA-PBS). After 3 washings with EDTA-PBS, platelets were counted and suspended in EDTA-PBS at a concentration of  $3 \times 10^8$ /ml.

Direct immunofluorescence test: Fifty microliters of platelet suspension were incubated with 1:50 diluted FITC conjugated goat anti-human immunoglobulin (IgG, IgM, IgA, kappa, lambda, F(ab')<sub>2</sub>, DAKO, Denmark) for 60 min in a dark place at room temperature, followed by 2 washings with EDTA-PBS containing 1% bovine serum albumin. Platelets were suspended in 100  $\mu$ l of PBS containing 25% glycerin for examination by fluorescence microscopy and diluted with EDTA-PBS at  $5-10 \times 10^5$ /ml for analysis by flow cytometry (FACS IV, Becton Dickinson, USA).

On fluorescence microscopy, results were expressed by (−): negative, (±): 0–1 fluorescence spot in a platelet, (+): a few fluorescence spots, (++): several fluorescence spots, (+++): numerous fluorescence spots, and dubious (d): few platelets were seen, or fluorescence was indistinguishable from non-specific or artifacts. In some cases, determination of fluorescence on microscopy was followed by FACS analysis. Fluorescence intensity was expressed by Rf value (relative fluorescence intensity) as follows.

$$\frac{\text{mean fluorescence channel of patient platelets}}{\text{mean fluorescence channel of normal platelets}} = \text{Rf value}$$

### Indirect immunofluorescence test

Preparation of normal PFA fixed platelets: Fifty

microliters of blood was collected from a normal healthy type 0 blood subject. PFA-fixed washed platelet suspension was prepared by the same procedure as described in the direct immunofluorescence test.

Indirect immunofluorescence test: Fifty microliters of PFA-fixed platelets were incubated with equal volumes of patient serum for 60 min at room temperature in a dark place. After 3 washings with EDTA-PBS, platelets were pelleted. Platelet pellet was re-suspended in 100  $\mu$ l of 1:100 diluted FITC conjugated goat anti-human immunoglobulin and incubated at room temperature for 60 min. Platelets were examined on fluorescence microscopy in the same fashion as in the direct immunofluorescence test.

### Indirect immunofluorescence test with eluted antibodies

Washed platelets (non-fixed) from ITP subjects were prepared as described in direct PSIFT. Washed platelets were suspended in 1 ml of 0.2% BSA-PBS. Platelet associated antibodies were eluted by lowering pH at 3.0 by 1 N HCl for 5 min. Platelets were removed during centrifugation and pH were adjusted to 7.2 by 1 M Na<sub>2</sub>HPO<sub>4</sub>. Indirect immunofluorescence test was carried out as described above.

## 3) PA-IgG assay by solid-phase competitive ELISA<sup>42)</sup>

### Preparation of IgG-coated assay plate

Purified human IgG (MBL, Nagoya) diluted with 0.1 M carbonate buffer at pH 9.6 at a concentration of 4  $\mu$ g/ml was incubated in each wells of microtiter assay plate (Falcon 3912, Becton Dickinson, USA) at 37°C for 2 hours, then washed 2 times with PBS. Residual protein binding was blocked by incubation with 1% BSA-PBS at 37°C for 2 hours. After 3 washings, assay plates were stored at 4°C up to one week.

### Preparation of sample platelets

Patient's blood was collected in a disposable plastic syringe containing 10% V/V 3.8% sodium citrate and kept at room temperature. Separation of platelets was started within 3 hours after sampling. When peripheral platelet count of the patient was less than  $3 \times 10^4/\mu$ l, 20 ml of blood was necessary for sufficient yield. PRP was separated by centrifugation at 150g, 22°C for 15 min. Ten percent V/V of ACD-A solution was added to PRP, then platelets were washed 4 times with EDTA-PBS. Platelets were suspended at a concentration of  $4 \times 10^8$ /ml and lysed by mixing with equal volume of EDTA-PBS containing 1% Triton X-100.

### PA-IgG assay

Fifty microliters of platelet lysate was incubated at 37°C for 2 hours with 50  $\mu$ l of 1:2,000 diluted alkaline phosphatase conjugated goat anti-human IgG, F(ab')<sub>2</sub>, (TAGO, USA) in each well of IgG-coated microtiter plate. Likewise, 12 dilutions of

standard IgG (0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 100, 250, 500, 1,000 ng/50  $\mu$ l) and no IgG (blank) were incubated. Samples and standards were assayed in duplicate. After 6 washings with PBS containing 0.05% Tween 20 (PBS-T), 50  $\mu$ l of alkaline phosphatase substrate (p-nitrophenyl phosphate tablet (Sigma, USA) dissolved in substrate buffer, pH 9.8, consisting of 97 ml of diethanolamine, 60 ml of 1 N HCl, 100 mg of MgCl<sub>2</sub>, 200 mg of NaN<sub>3</sub> and 843 ml of water) were added to each well and incubated at 37°C for 10 min. The reaction product, nitrophenolate, was measured at 405 nm by an ELISA reader (EAR-400, SLT-Labo instruments, Austria). PA-IgG was calculated using a standard curve and represent "ng" of IgG per 10<sup>7</sup> platelets.

#### 4) PB-IgG assay and antibodies to platelet component lipids

##### PB-IgG assay

PB-IgG was measured using the modification of ELISA by Schiffer et al<sup>35</sup>. Washed platelets fixed with PFA were prepared as described by the indirect immunofluorescence test and suspended at a concentration of 3 × 10<sup>7</sup>/ml in EDTA-PBS. Then, 100  $\mu$ l of platelet suspension was seeded in microtiter wells, which had been previously coated with 70  $\mu$ g/ml of poly-L-lysine (Sigma, USA) for 30 min at room temperature. The assay plate was centrifuged at 1,500g for 10 min. After blocking with 1% BSA-PBS at 37°C for 1 hour, 50  $\mu$ l of 1:16 diluted sample serum was added to each well and incubated at 37°C for 3 hours, then washed 6 times with PBS-T. The microtiter plate was incubated with 1:1,000 diluted alkaline phosphatase conjugate anti-human IgG at 37°C for 45 min, washed 6 times. Alkaline phosphatase substrate was added to each well of microtiter plate for 10 min as described in the PA-IgG assay, and the substrate products were measured at 405 nm by an ELISA reader.

##### Anti-cardiolipin antibody and anti-sulfatides antibody assay

The levels of anti-cardiolipin antibody and anti-sulfatides antibody were measured according to a modification of ELISA by Harris et al<sup>16</sup>.

Fifty microliters of cardiolipin and sulfatides (each 50  $\mu$ g/ml) in ethanol were incubated in microtiter wells and dried in cold air flow for 30 min. The microtiter plate was blocked by 1% BSA-PBS after washing with PBS. Fifty microliters of 1:32 diluted patient serum was incubated in microtiter wells at 37°C for 3 hours, then washed 3 times with PBS-T. The microtiter plate was incubated with 1:1,000 diluted alkaline phosphatase conjugated anti-human IgG antibody at 37°C for 45 min. After 6 washings with PBS-T, the amount of antibody binding was determined as described in PB-IgG assay.

#### 5) Immunoprecipitation

Samples were 19 patients with ITP which was positive in indirect PSIFT (Positive Samples in Indirect Immunofluorescence test of this study were not included). Immunoprecipitation was performed according to the method of Anderson et al<sup>2</sup>.

##### Preparation of <sup>125</sup>I-labeled platelets

Washed platelets (1 × 10<sup>9</sup>) were prepared from normal subjects, then suspended with citrate glucose buffer (CGS: 150 mM NaCl, 40 mM sodium citrate, 0.6% dextrose, pH 6.8–7.0). Lactoperoxidase (1.4 × 10<sup>-4</sup> M) followed by Na-<sup>125</sup>I (0.8 mCi) was added to washed platelets with shaking. Then, 10  $\mu$ l of H<sub>2</sub>O<sub>2</sub> (10<sup>-2</sup> M) was added 5 times at interval of 20 seconds. Platelets were washed 3 times with CGS, then suspended in 20 ml of Tris buffered saline containing 1 mM Ca<sup>++</sup> (150 mM NaCl, 20 mM Tris, 1 mM CaCl<sub>2</sub>, pH 7.2).

##### Immunoprecipitation and SDS-PAGE

Two hundred microliters of <sup>125</sup>I-labeled platelets (1 × 10<sup>8</sup>) were incubated with equal volumes of patient serum for 1 hour at room temperature, then washed 3 times with CGS. Platelet pellet was lysed with 1% Triton X-100 in EDTA-PBS, and residual platelet components were removed during centrifugation at 12,000g for 10 min. To platelet lysate, 1/6 volumes of 10% Staphylococcal Protein A (IgG Sorb, The Enzyme Center, USA) was added and incubated with shaking for 1 hour. Protein A was previously absorbed by non-labeled platelet lysate to reduce non-specific platelet protein bindings. After 3 washings, Protein A with immune complexes, was solubilized in SDS sample buffer (62.5 mM Tris, 10% Glycerol, 2% SDS, 5% V/V 2-mercaptoethanol, 0.008% bromophenol blue (BPB) in final concentration) in a reduced condition and electrophoretically separated in 7.5% SDS-PAGE. The gels were then fixed and stained overnight with Coomassie brilliant blue (0.025% brilliant blue, 45% methanol, and 3% acetic acid in distilled water), dried, and applied to X-ray film and exposed for 2 weeks.

#### 6) Western blotting analysis

Western blotting analysis was performed according to a modification of the method by Lynch et al<sup>27</sup>.

##### Platelet protein electrophoresis

Washed whole platelets (5 × 10<sup>8</sup>) were solubilized in 1.0 ml of SDS sample buffer (62.5 mM Tris, 10% glycerol, 2% SDS, 0.0125% N-acetyl maleimide, 0.008% BPB in final concentration) and heated 10 mins in a boiling water bath. Platelet proteins were separated for 17 hours at 50 volts on 7.5% polyacrylamide gel in a non-reduced condition. Following this, platelet proteins were electrophoretically transferred to a nitrocellulose membrane (0.45  $\mu$ m, Trans-Blot Transfer Medium, Bio-Rad, USA) at 450 mA for 4 hours in Trans-Blot Cell (Bio-Rad, USA) with 10 mM sodium tetraborate buffer, pH 9.2. The polyacrylamide gels with molecular markers were

stained for proteins with Coomassie brilliant blue. Proteins transferred on a nitrocellulose membrane (platelet protein and molecular markers) were also stained with colloidal gold (Auro-Dye, Janssen Life Science, Belgium).

### Immunostaining

Nitrocellulose membrane with platelet proteins were washed 3 times with Tris washing solution (TWS: 10 mM Tris, 150 mM NaCl, 0.05% Nonidet P-40, 0.01% SDS, pH 7.0). Residual protein binding sites on the membrane were blocked by 5% gelatin in TWS at 37°C for 1 hour, followed by 50% bovine serum in TWS for 1 hour. The membrane was incubated with 1:10 diluted patient serum at 37°C for 2 hours with shaking in a leak-free incubation device (Screener blotter, Samplatec, Osaka), then, washed 4 times with TWS. To detect immunoglobulin bindings, horseradish peroxidase (HRP) conjugated goat anti-human IgG (EIA blotting grade, Bio-Rad, USA) 1:2,000 diluted with TWS was incubated with the membrane with shaking at 37°C for 1 hour, and washed 6 times with TWS. IgG bindings were visualized by high sensitivity HRP substrate (Konica immunostain HRP, Konica, Tokyo). Approximate molecular weight of the proteins was determined using a high molecular weight standard (Bio-Rad, USA) on a polyacrylamide gel stained by Coomassie brilliant blue and on a nitrocellulose membrane stained with colloidal gold.

## RESULTS

### 1) Platelet suspension immunofluorescence test (PSIFT)

#### Direct immunofluorescence test

Under fluorescence microscopy, 44 ITP subjects in various clinical conditions were examined. Twenty eight were negative, 5 were ( $\pm$ ), 7 were (+), 2 were (++), one was (+++) and one was (d) for IgG+A+M antibody. Positive results of more than ( $\pm$ ) were noted in 15 ITP subjects (34.1%). Fluorescence intensity was also examined by flow cytometry. Fig. 1 shows typical fluorescence histograms in control subjects and positive ITP patient. Rf values on flow cytometric analysis correlated well with the semi-quantitative results of fluorescence microscopy (Fig. 2).

#### Indirect immunofluorescence test

In 48 ITP subjects, only 2 cases (4.2%) were positive for IgG+A+M antibody, ( $\pm$ ) and (++). Flow cytometric analysis was not performed by the indirect method.

#### Indirect immunofluorescence test with eluted antibodies

Platelet associated antibodies were eluted from 18 direct PSIFT positive ITP patients by acidification method. Indirect test for eluted antibodies was positive in 3 cases (16.7%) for IgG+A+M antibody, ( $\pm$ ) in 1 case and (+) in 2 cases.

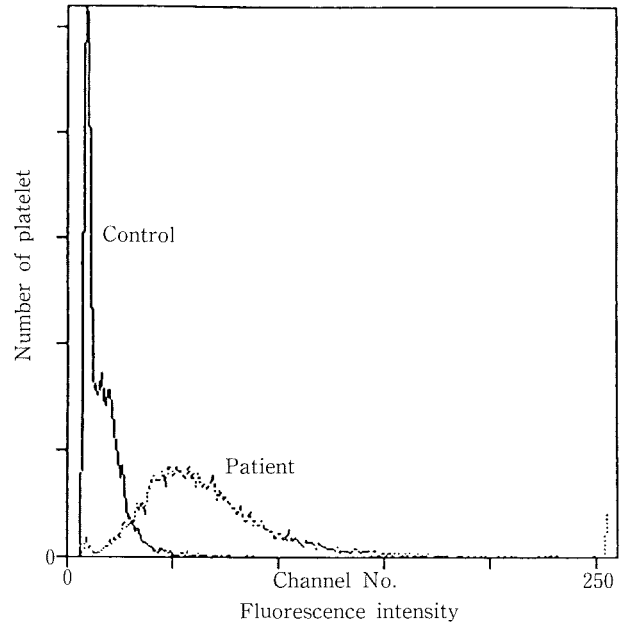


Fig. 1. Fluorescence histograms of control subject and positive patient on flow cytometric analysis.

Mean fluorescence channel was 16.2 in control subject and 68.7 in the patient. Rf value was 4.24. Fluorescence was (+++) on fluorescence microscopy.

Rf value (FACS)

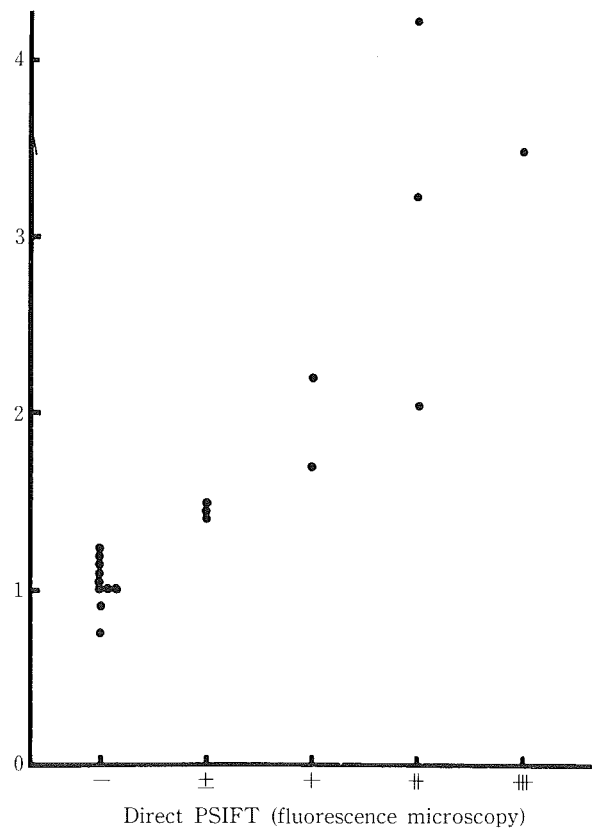
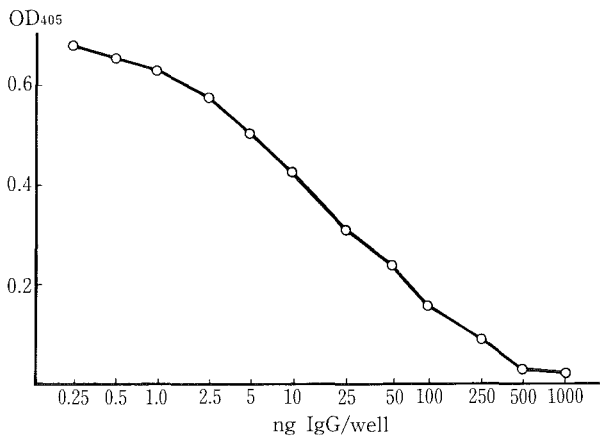


Fig. 2. Correlation between Rf values on FACS analysis and semi-quantitative result on fluorescence microscopy.

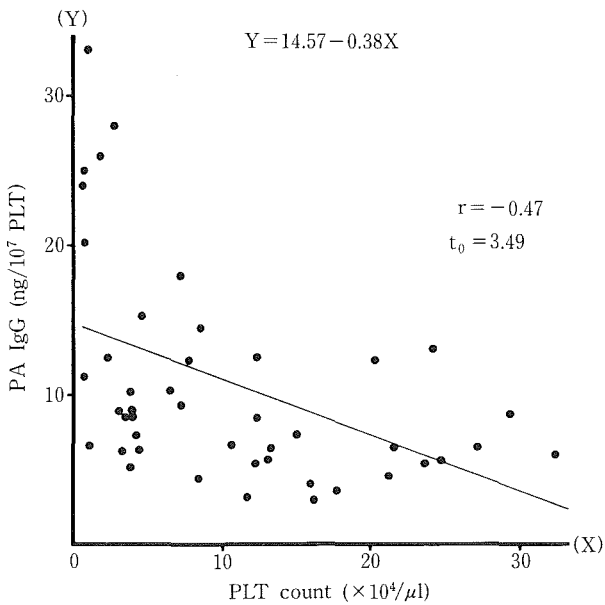
Rf value correlated well with the result of PSIFT under microscopy.

Rf value: relative fluorescence

PSIFT: platelet suspension immunofluorescence test



**Fig. 3.** Standard titration curve in PA-IgG assay using competitive ELISA.  
PA-IgG: calculated from the curve between 2.5 and 100 ng IgG.



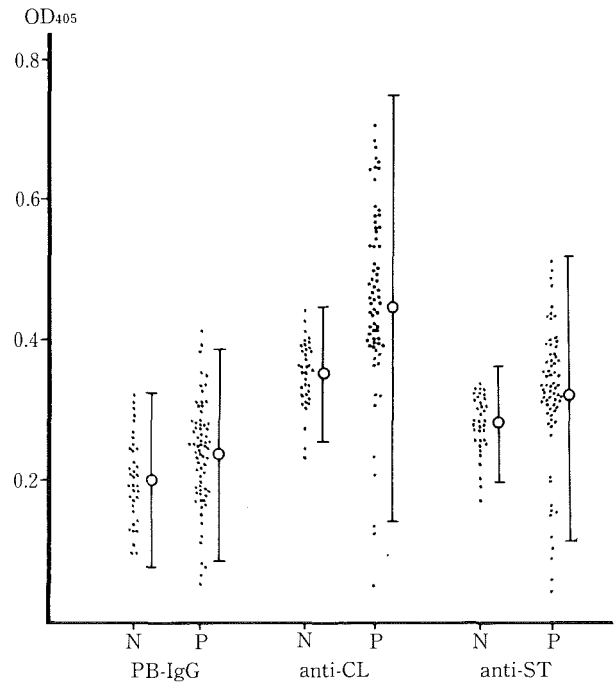
**Fig. 4.** Correlation between PA-IgG and platelet count in 45 chronic ITP subject.  
Inverse correlation between PA-IgG and platelet count noted ( $p < 0.01$ ).

**2) PA-IgG assay by solid-phase competitive ELISA**

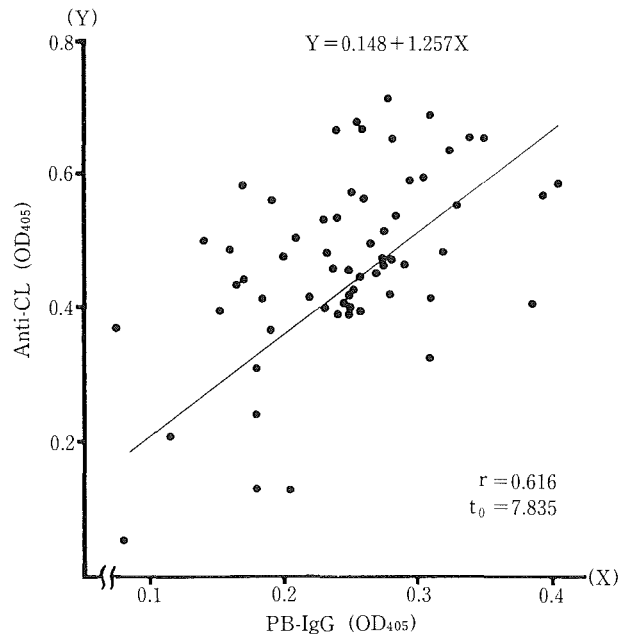
Fig. 3 shows the standard curve in this assay. PA-IgG levels in 14 normal subjects were  $4.5 \pm 4.4$  ng/ $10^7$  platelets (mean  $\pm$  2SD). Elevation of PA-IgG more than 9.9 ng/ $10^7$  platelets (mean  $\pm$  2SD) was noted in 17 among 45 ITP subjects (37.8%). Fig. 4 shows the relationship between PA-IgG and peripheral platelet counts. An inverse correlation between PA-IgG and platelet counts was noted (t-test,  $p < 0.01$ ).

**3) PB-IgG and antibodies to platelet component lipids**

Sixty five patients with ITP and 36 normal sub-

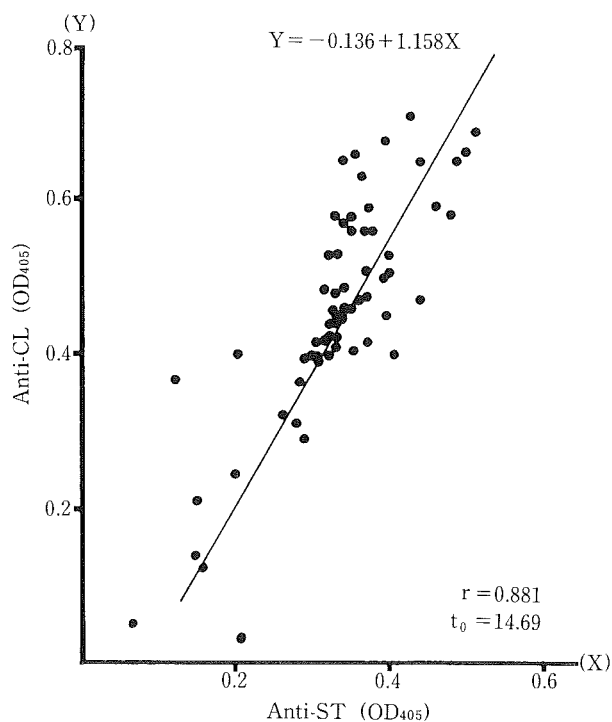


**Fig. 5.** PB-IgG, anti-cardiolipin antibody and anti-sulfatides antibody in 65 chronic ITP subjects.  
PB-IgG: platelet bindable IgG  
anti-CL: anti-cardiolipin (IgG) antibody  
anti-ST: anti-sulfatides (IgG) antibody



**Fig. 6.** Correlation between PB-IgG and anti-cardiolipin antibody in chronic ITP subjects.  
Positive correlation noted ( $p < 0.01$ ).

jects were examined. Absorbance at 405 nm ( $OD_{405}$ ) in 36 normal subjects were  $0.203 \pm 0.121$  (mean  $\pm$  2SD) in PB-IgG,  $0.352 \pm 0.095$  in anti-cardiolipin antibody,  $0.282 \pm 0.086$  in anti-sulfatides antibody. In ITP subjects,  $OD_{405}$  were



**Fig. 7.** Correlation between anti-cardiolipin antibody and anti-sulfatides antibody in chronic ITP subjects.

Significant correlation noted ( $p < 0.001$ ).

$0.243 \pm 0.153$  in PB-IgG,  $0.453 \pm 0.312$  in anti-cardiolipin antibody,  $0.327 \pm 0.199$  in anti-sulfatides antibody. Positive results of PB-IgG, anti-cardiolipin antibody and anti-sulfatides antibody more than two standard deviations from normal subjects, were noted in 8 (12.5%), 36 (56.3%), and 20 (31.3%), respectively (Fig. 5).

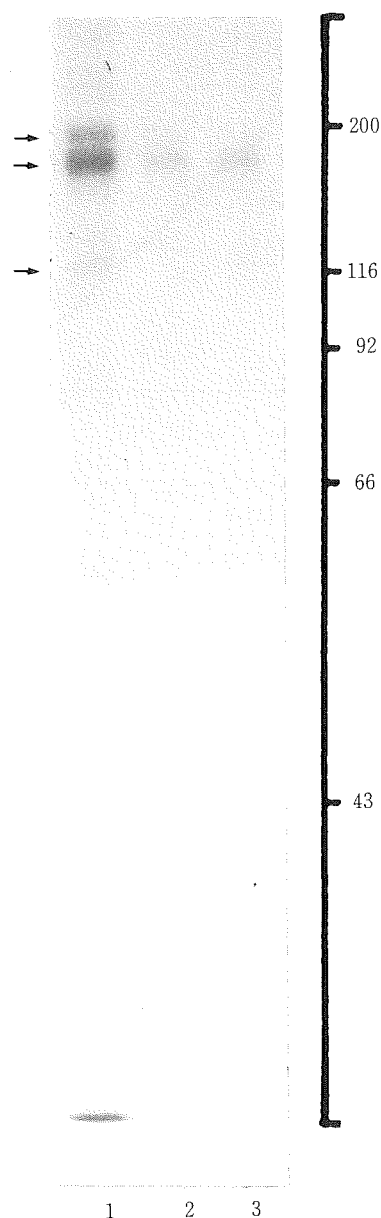
Positive correlation between PB-IgG and anti-cardiolipin antibodies ( $t$ -test,  $p < 0.01$ ), anti-cardiolipin antibodies and anti-sulfatides antibodies ( $t$ -test,  $p < 0.001$ ), which were assayed simultaneously, was noted (Fig. 6 and Fig. 7). Correlation between platelet count and PB-IgG, platelet count and anti-cardiolipin antibody or anti-sulfatides antibody were also examined, but no correlation was found.

#### 4) Immunoprecipitation

Anti-platelet antibodies were precipitated in one out of 19 ITP patients. Fig. 8 shows the autoradiography of immunoprecipitation in this patient (lane 1). Approximate molecular weight of these antigens was 190 kd, 170 kd and 115 kd under a reduced condition. Antigen of approximate molecular weight 170 kd was strongly precipitated.

#### 5) Western blotting analysis

Target platelet antigens against anti-platelet antibody (IgG) were detected in six out of 48 patients with ITP (Fig. 9). Table 1 presents representative antigen distributions of six patients. Four patients had splenectomized (Case 1, 4, 5 and 6). Platelet count was less than  $3 \times 10^4/\mu\text{l}$  in four cases (Case



**Fig. 8.** Autoradiogram of  $^{125}\text{I}$ -radiolabeled platelets that bind to IgG antibodies in one patient with ITP (lane 1) and control subject (lane 2–3).

190 kd, 170 kd and 115 kd proteins precipitated.

2–5). A further patient had  $11.6 \times 10^4/\mu\text{l}$  of platelet, but purpura did not vanish after splenectomy. Non-specific IgG binding to some proteins could not be excluded in these conditions. IgG binding to molecular weight 55 kd and 72 kd proteins were noted in normal subjects.

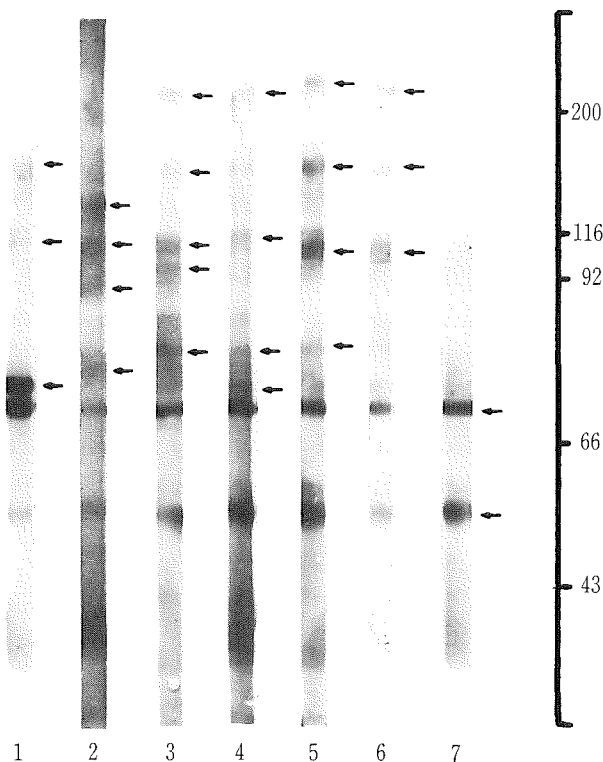
## DISCUSSION

### 1) Outline of anti-platelet antibodies

Many assay techniques which detect various types of anti-platelet antibodies have been developed. Table 2 shows the most common detection methods of anti-platelet antibodies. Because of their low sensitivity in detecting auto-antibodies, methods other than direct quantification of immunoglobulin are not

**Table 1.** Antigen distribution and patient profiles in six patients with ITP

Case No.	Approximate molecular weight (Kd)						platelet count ( $\times 10^4/\mu\text{l}$ )	PA-IgG (ng/ $10^7$ plt)	Remarks	
1	155		105				75	17.7	3.7	Post splenectomy
2		135	110		90	78		3.1	8.9	
3	210			105	100		80	3.5	8.5	Drug free
4	210		110				80	0.7	24.0	Post splenectomy
5	220			105			80	0.8	20.2	Evans syndrome, Post splenectomy
6	220	155		105				11.8	3.2	Post splenectomy



**Fig. 9.** Western blotting results of solubilized platelets that bind to IgG antibodies in six patients with ITP (lane 1–6. Lane number is identical to case number in Table 1) and control (lane 7) subject.

commonly used today. Immunoglobulin, which was assayed by various methods, may be divided into two categories. One consists of platelet bindable antibody in serum which will bind to platelets. Another is platelet associated antibody which is bound on platelets or exist in platelets. Now, an inverse correlation between PA-IgG and platelet count in ITP subjects has been demonstrated and the utility of PA-IgG in the diagnosis of ITP has been well recognized.

## 2) Platelet associated antibodies

A simple detection technique for platelet associated immunoglobulin was established by von dem Borne and colleagues<sup>5,6</sup>. Their simple immunofluorescence test can semi-quantitatively detect surface bound IgG, IgA and IgM, separately or simultaneously. It has been used for ordinary

laboratory examination of allo- and auto-antibodies. The direct PSIFT used in this study showed an overall positive rate of 34.1% in ITP patients including refractory cases and complete remission cases. On direct PSIFT, we also attempted to quantify the fluorescence intensity by flow cytometry. Fluorescence, which was judged on microscopy, correlated well with the Rf values. These data indicate that flow cytometric analysis is useful in quantification of fluorescence intensity in PSIFT. However, we still have difficulty in expressing IgG quantity by “grams of IgG per platelet” or “molecules of IgG per platelet”.

Previously, Takamatsu reported PA-IgG assay using rocket immunoelectrophoresis<sup>40</sup>. Another platelet associated immunoglobulin assay is ELISA which enables quantitative measurement IgG and it is widely used in Japan. PA-IgG assay using competitive ELISA used in this study was more sensitive and needs less time than rocket immunoelectrophoresis. PA-IgG was correlated well with the degree of thrombocytopenia of ITP patients in this study, as previously reported<sup>23,41</sup>. However, there are discrepancies in the normal range of PA-IgG among many assay methods. These discrepancies may come from, in part, different treatment procedures of sample platelets<sup>25</sup>. Platelets contain immunoglobulin on their surface and inside. Therefore, the total PA-IgG, which was measured with platelet lysate, was usually higher than surface PA-IgG<sup>24</sup>. Solubilizing agents such as Triton X-100 at high concentration may also interfere with immunoglobulin binding. Another possibility is the liberation of surface and/or total immunoglobulin which was noted in the platelet aggregation and activation which may occur during the washing procedure<sup>33</sup>. The washing procedure by PBS, containing EDTA, is a standard method. Quality of labeled antiserum is also an important factor. It is known that PSIFT may show conflicting results due to differences in anti-serum among procedures. Immunoglobulin class, IgG solely or total IgG+A+M, is also a problem. Correlation between direct PSIFT (IgG+A+M) and PA-IgG in the same samples was poor (data not shown). Because of the elevation of platelet associated immunoglobulin other than IgG, PA-IgG does not correlate with the result of direct PSIFT

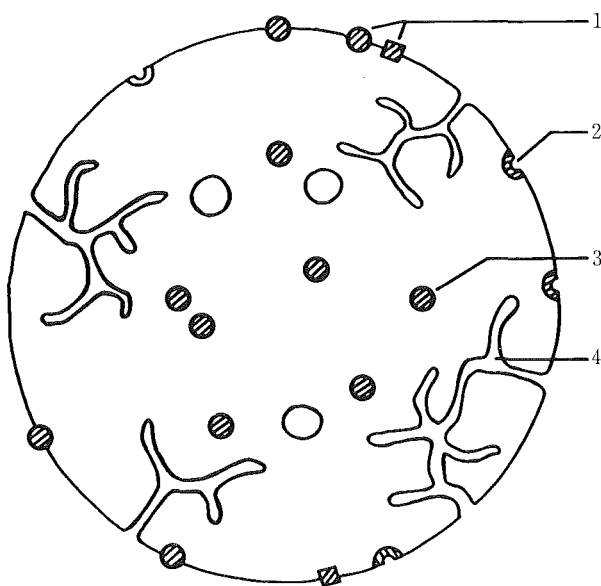
**Table 2.** Detection techniques of anti-platelet antibodies

Report	Detection techniques	Type of antibodies
1954 Flucker	platelet Coombs test	platelet agglutinin
1960 Steffen	antiglobulin consumption test	PB-IgG, PA-IgG
1969 Aster	<sup>51</sup> Cr PNH platelet release test	PB-IgG
1969 Karpatkin	platelet factor 3 test	PB-IgG
1971 McMillan	<sup>131</sup> I Fab-anti F(ab) <sub>2</sub> system	PA-IgG
1973 Hirschman	<sup>14</sup> Cr-Serotonin release test	PB-IgG
1975 Dixon	complement lysis-inhibition assay	PA-IgG
1975 Van Boxtel	fluorescein	PB-IgG
1977 Tate	immunoenzyme histochemical method	PB-IgG
1977 Luiken	<sup>125</sup> I-Fab anti-Fab test	PA-IgG
1978 von dem Borne	PSIFT	PA-IgG, PB-IgG
1979 Cines	<sup>125</sup> I Coombs test	PA-IgG, PA-C <sub>3</sub>
1979 Hymes	solid phase RIA Staph. protein A	PA-IgG
1979 Leporrier	immunoperoxidase method	PA-IgG
1980 Sugiura	fluorospectrophotometry	PA-IgG
1980 Nel	ELISA	PA-IgG, PA-C <sub>3</sub>
1981 Hedge	enzyme linked immunoassay	PA-IgG
1981 Morse	rocket immunoelectrophoresis	PA-IgG
1986 Lazarchick	flow cytometry	PA-IgG

PA-IgG : platelet associated immunoglobulins

PB-IgG : platelet bindable immunoglobulins

PA-C<sub>3</sub> : platelet associated complement (C<sub>3</sub>)



**Fig. 10.** Locations of platelet associated antibodies in platelet.

1. Bound to platelet specific antigens by Fab domain
2. Bound to Fc receptor
3. Located in alpha granules or phagosome
4. Located in open canalicular system

(IgG + A + M) in some cases. Which is superior, IgG or total IgG + A + M, has not been conclusively established. On the other hand, elevation of PA-IgG was reported in systemic lupus erythematosus (SLE), Sjögren's syndrome, drug-induced thrombocytopenia, liver cirrhosis, myelodysplastic syndrome (MDS) and many other diseases with or without thrombocytopenia<sup>14,23</sup>. We also found that direct PSIFT or PA-IgG was positive in SLE, Sjögren's

syndrome, liver cirrhosis and MDS in rocket immunoelectrophoresis<sup>41</sup> and competitive ELISA. In short, elevation of PA-IgG in various assays showed high sensitivity, but low specificity for ITP.

PA-IgG is considered to consist of platelet specific antibody and other non-specific antibodies. Fig. 10 shows the estimated locations of platelet associated immunoglobulin. Normal platelets contain a small amount of plasma proteins, which include immunoglobulin, in the open canalicular system, rather like a sponge<sup>1</sup>. IgG was also found in alpha-granule of normal platelets<sup>13</sup>. Endressen et al. showed that pepsin treated anti-platelet F(ab)<sub>2</sub> antibody did not bind to normal platelets, and they estimated that anti-platelet antibody in SLE is bound to platelets as an immune complex by Fc receptor<sup>12</sup>. In order to demonstrate platelet specific autoimmunity, it is necessary to determine the target platelet antigens. Techniques of binding site evaluation are discussed below.

### 3) Platelet bindable antibodies

Circulating anti-platelet antibodies have been considered to be less important than platelet associated antibodies, because correlation with thrombocytopenia, both positive in a small population of ITP patients and also in allo-immunization, was not established. In this study, the rate of positive cases was only 4.2% in indirect PSIFT and 12.5% in PB-IgG assay using ELISA. Acid eluted immunoglobulins, which were thought to be bound by the Fab portion, were also positive in only 3 out of 18 ITP patients. These results showed a low positive rate in ITP. Likewise, other PB-IgG assays, the ELISA technique described in this report



**Table 3.** Previously reported target antigens

Report	Target antigens	Frequency	Detection techniques
1982 van Leeuwen et al.	GPIIb/IIIa	32/42	PSIFT
1984 Beardsley et al.	GPIIIa	9/13	Western blotting
1984 Woods et al.	GPIIb/IIIa	5/56	Immunoprecipitation
1984 Woods et al.	GPIb	3/106	Immunoprecipitation
1984 Devine et al.	GPIIb/IIIa	1/7	Immunoprecipitation
1984 Anderson et al.	GPIIb/IIIa	1/1	Immunoprecipitation
1984 Mason et al.	Multiple	3/5	Western blotting
1986 Szatkowski et al.	GPIb	1/1	CIE
1986 Lynch et al.	Multiple	21/23	Western blotting
1987 Beardsley	GPIb	2/3 Acute ITP	Western blotting
	100kd(GPIIIa)	32/48 Chr. ITP	Western blotting
	85kd	7/7 Post VZ ITP	Western blotting
1987 McMillan et al.	GPIb	23/59	Immunobeads & ELISA
	GPIIb/IIIa	13/59	Immunobeads & ELISA

PSIFT : platelet suspension immunofluorescence test

CIE : crossed immunoelectrophoresis

had a number of methodological problems. (1) Normal platelets, used as the antigen, contained a small amount of immunoglobulin. This may cause the background. (2) The number of platelets which can be fixed in a microtiter well is limited. Consequently, the antigens are a small component of platelets, and not sufficiently numerous to be detectable. (3) Allo-antibodies which are directed mainly to HLA antigens are indistinguishable from auto-antibodies. (4) Immune complex or aggregated immunoglobulin, which bind to platelets by platelet Fc receptor, may be found. These constitute unresolved problems in the PB-IgG assay.

#### 4) Target platelet antigens

Platelet antigens have been classified into allo-antigens, iso-antigens, auto-antigens, cryptic antigens, xeno-antigens and haptens<sup>7,8)</sup>. Allo-antigens have been regularly identified as Zw(PI<sup>A</sup>), PI<sup>E</sup>, Ko, Bak, and recently found Yuk. However, auto-antigens had not been disclosed for over a decade, because auto-antibodies are very weak in ITP. van Leeuwen et al<sup>26)</sup> first reported GP IIB/IIIa as a target antigen. They examined serum and eluates of ITP patients. The serum was positive in indirect PSIFT and the eluates were prepared from platelets of ITP subjects. Both reacted with normal type 0 platelets, but did not react with platelets of Glanzmann's thrombasthenia which defects GP IIB/IIIa. To date, GP IIB/IIIa<sup>2,9,30,45)</sup>, GP IIIa<sup>3,4)</sup> and GP Ib<sup>4,30,39,46)</sup> have been reported as target antigens (Table 3).

Several methods have been used to detect target antigens (Table 3): Primarily, immunoprecipitation and Western blotting.

#### Immunoprecipitation Method

In the immunoprecipitation method, anti-platelet antibodies bind to intact antigens on platelet surface. "Intact antigen" is an important factor for detecting conformational antigenic sites, such as GP IIB/IIIa complex. The immunoprecipitation method

has a number of advantages. By the lactoperoxidase method, platelet surface proteins, especially glycoproteins, are not radiolabeled uniformly. Whereas GP IIB and IIIa are strongly labeled. GP Ib, GP IIIb, GP V, which contain a large amount of carbohydrate, are labeled poorly. The rate of radiolabeling of each protein is also variable in other methods, such as diazodiodosulfanilic acid labeling, and periodate [<sup>3</sup>H]NaBH<sub>4</sub> labeling. Affinity of Protein A is another problem. Protein A strongly binds IgG<sub>1</sub>, IgG<sub>2</sub> and IgG<sub>4</sub>, but does not bind others. Therefore, immunoprecipitation with <sup>125</sup>I labeled platelets by the lactoperoxidase method, also used in this study, is valuable for detecting IgG (except IgG<sub>3</sub>) antibodies to GP IIB/IIIa complex, GP IIB, and GP IIIa. However, the detection rates by immunoprecipitation of anti-GP IIB and/or IIIa antibody reported by many authors are not high compared to allo-antibody detection<sup>9)</sup>. Thus, the main problem with the immunoprecipitation method in the detection of platelet auto-antigens is degree of sensitivity.

#### The Western Blotting Technique

By contrast, Western blotting has a higher detection rate than the immunoprecipitation method in recent studies. The Western blotting technique in platelet serology has several characteristics that must be taken into account.

(1) Auto-antibodies in chronic ITP are very weak compared to allo-antibodies. Even in low serum dilution and long incubation time (background bindings may increase in these conditions), target antigens may not be detected in most of the ITP serum. If the reacting conditions are severe, for example in high salt concentration (e.g. 0.5 M NaCl), few auto-antibodies were bound to the membrane in this study. On the contrary, a very small amount of antigen might exist in platelets. If the target antigen is a trace component of platelets, the amount of antigen on nitrocellulose membrane may be too small to be detected. These problems were dis-

cussed in detail by Huisman<sup>19</sup>). At any rate, it is not easy to determine the positivity or negativity in weakly stained bands on the nitrocellulose membrane.

(2) Loss of conformational antigenic sites, which is the major problem, may occur during solubilization and electrophoresis. Many antigens bind allo-antibodies in a non-reduced condition but did not react in a reduced condition. Anti-GP IIb/IIIa complex antibody could not be detected with the Western blotting method, because GP IIb and IIIa were dissociated during SDS PAGE.

(3) Non-specific IgG binding is another problem. In this study, non-specific IgG bindings to 72 kd protein was probably due to Fc gamma receptors in monocytes contaminated in platelet lysate. Monocytes have been reported to have 40 kd and 72 kd Fc gamma receptors. Many IgG receptors on platelets were also reported, 40 kd protein<sup>34</sup>, 210 kd glycoprotein<sup>38</sup>, and GP IIIa<sup>36</sup>. IgG may binds non-specifically to IgG receptors and in a part specifically to these proteins as target antigen.

(4) There is the possibility that IgG binds to antigens which do not exist on the surface of intact platelets. These antigens may be degenerated products of platelet membranes, platelet membrane internal proteins or platelet cytoplasmic proteins.

#### The Significance of Target Platelet Antigens

Until recently, we have little knowledge about the significance of target antigens. Beardsley et al. reported that GP Ib was frequent in acute ITP, 100 kd protein (GP IIIa) was frequent in chronic ITP and 85kd protein were frequent in post Varicella-Zoster ITP. They also found that the patient with anti-GP IIIa antibody had a severe bleeding tendency. In this study, multiple antigenic binding sites were detected in six patients. The presence of multiple binding sites in Western blotting was also reported<sup>27,28</sup>. In these reports, the molecular weight of the binding sites ranged from 45 kd to 255 kd and more than 20 binding sites were noted. Both the results of this study together with these reports suggest the presence of heterogeneous antibodies against various antigens, or homogeneous antibodies against specific antigenic epitope which is present on different platelet proteins, the so-called common antigenic site. Meanwhile, it is not easy to determine the identity of antigen by molecular weight. For example, the molecular weight of GP IIIa is variable depending on the conditions of SDS-PAGE. To confirm the identity of GP IIIa, immunoglobulin bindings must be compared with the binding to platelets from patients with Glanzmann's thrombasthenia which defects GP IIb/IIIa. In this stage, it is better to express target antigens simply by their molecular weight. A high incidence of post splenectomy state (four out of 6 cases) was noted in Western blotting of this study. Additional Western blotting result showed stronger bands on pre splenectomy state than on

post splenectomy state in Case 6. It is thought that immune abnormalities still existed after splenectomy in this case.

Target antigens other than protein were also estimated. Anti-cardiolipin antibody was first reported to correlate with platelet count in SLE by Harris et al<sup>17</sup>). However, the correlation was not noted in ITP<sup>18</sup>). Anti-cardiolipin antibody was detected in about half of the ITP patients in this study, but the correlation between platelet count and level of anti-cardiolipin antibody was not noted. The anti-cardiolipin antibody has less pathological significance in platelet destruction of ITP, and has a different meaning from that of SLE. Antibodies against glycosphingolipids, which are components of platelet lipids, were demonstrated by van Vliet et al<sup>44</sup>). Sulfatides, which is a trace platelet glycosphingolipids, was also estimated to be the target antigen. In this study, anti-sulfatides antibodies were also detected in one third of the ITP patients. A correlation between anti-cardiolipin antibody and PB-IgG suggests that one of the anti-platelet antibodies is directed against cardiolipin which is a component of platelet membrane. There was strong correlation between anti-cardiolipin antibody and anti-sulfatides antibody, which suggests the presence of common antigenic epitopes in these lipids.

Target platelet antigens in other diseases were recently disclosed. Kaplan et al. reported a 108 kd protein, which was located in the cytoplasmic fraction of the platelets, against anti-platelet antibody in SLE<sup>20</sup>). GP IIb in pseudothrombocytopenia<sup>43</sup>, pseudo-Bernard-Soulier syndrome in anti-GP Ib auto-antibody<sup>10</sup>), acquired thrombasthenia in anti-GPIIb/IIIa auto-antibody<sup>31</sup>), and 25 kd platelet membrane protein in homosexual men with thrombocytopenia<sup>37</sup>) were also reported.

Clinically, ITP is a well characterized disease entity, but immunologically the mechanism of auto-antibody production has not been disclosed. Target platelet antigen, which is commonly found in ITP or characterize ITP, has not been found. Target antigen distribution of ITP may variable from case to case. However, further evaluation of target antigen may provide a clue to sub-classification of ITP or prognostic factors, target antigen analysis.

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## REFERENCES

1. Adelson, E., Rheingold, J.J. and Crosby, W.H. 1961. The platelet as a sponge: A review. *Blood* **17**: 767–774.
2. Anderson, M.J., Woods, V.L., Tani, P.B.A., Lindstrom, J.M., Schmidt, D. and McMillan, R. 1984. Autoantibodies to platelet glycoprotein IIb/IIIa and acetylcholine receptor in a patient with chronic idiopathic thrombocytopenic purpura and myasthenia gravis. *Ann. Intern. Med.* **100**: 829–831.
3. Beardsley, D.S., Spiegel, J.E., Jacobs, M.M., Handin, R.I. and Lux, IV. S.E. 1984. Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. *J. Clin. Invest.* **74**: 1701–1707.
4. Beardsley, D.S. 1987. Immune thrombocytopenia (ITP): Platelet target antigens of the antibodies in different clinical settings. *Thromb. Haemost.* **58**: 531.
5. von dem Borne, A.E.G.Kr., Verheugt, F.W.A., Oosterhof, F., von Riesz, E., de la Riviere, A.B. and Engelfriet, C.P. 1978. A simple immunofluorescence test for the detection of platelet antibodies. *Br. J. Haematol.* **39**: 195–207.
6. von dem Borne, A.E.G.Kr., Helmerhorst, F.M., van Leeuwen E.F., Pegels, H.G., von Riez, E. and Engelfriet, C.P. 1980. Autoimmune thrombocytopenia: Detection of platelet autoantibodies with the suspension immunofluorescence test. *Br. J. Haematol.* **45**: 319–327.
7. von dem Borne, A.E.G.Kr., van der Lelie, H., Vos, J.J.E., van der Plas-van Dalen, C.M., Risseuw-Bogaert, N.J., Ticheler, M.D.A. and Pegels, H.G. 1986. Antibodies against cryptantigens of platelets. *Curr. Stud. Hematol. Blood Transfus.* **52**: 33–46.
8. von dem Borne, A.E.G.Kr. 1988. Immunological structures of the platelet membrane. *Curr. Stud. Hematol. Blood Transfus.* **55**: 32–44.
9. Devine, D.V. and Rosse, W.F. 1984. Identification of platelet proteins that bind alloantibodies and autoantibodies. *Blood* **64**: 1240–1245.
10. Devine, D.V., Currie, M.S., Rosse, W.F. and Greenberg, C.S. 1987. Pseudo-Bernard-Soulier Syndrome: Thrombocytopenia caused by autoantibody to platelet glycoprotein Ib. *Blood* **70**: 428–431.
11. Dixon, R., Rosse, W. and Ebbert, L. 1975. Quantitative determination of antibody in idiopathic thrombocytopenic purpura: Correlation of serum and platelet-bound antibody with clinical response. *N. Engl. J. Med.* **292**: 230–236.
12. Enderson, G.K.M. and Foerre, O. 1982. Studies on the binding of immunoglobulins and immune complexes to the surface of human platelets: IgG molecules react with platelet Fc receptors with the CH<sub>3</sub> domain. *Int. Arch. Allergy Appl. Immunol.* **67**: 33–39.
13. George, J.N., Saucerman, S., Levine, S.P. and Knieriem, L.K. 1985. Immunoglobulin G is a platelet alpha granule-secreted protein. *J. Clin. Invest.* **76**: 2020–2025.
14. Hall, A.G., Proctor, S.J. and Saunders, P.W.G. 1987. Increased platelet associated immunoglobulin in myelodysplastic syndromes. *Br. J. Haematol.* **65**: 245–246.
15. Harrington, W.J., Minnich, V., Hollingsworth, J.W. and Moore, C.V. 1951. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J. Lab. Clin. Med.* **38**: 1–10.
16. Harris, E.N., Gharavi, A.E., Boey, M.L., Patel, B.M., Mackworth-Young, C.G., Loizou, S. and Hughes, G.R.V. 1983. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* **2**: 1211–1213.
17. Harris, E.N., Asherson, R.A., Gharavi, A.E., Morgan, S.H., Derue, G. and Hughes, G.R.V. 1985. Thrombocytopenia in SLE and related autoimmune disorders: association with anticardiolipin antibody. *Br. J. Haematol.* **59**: 227–230.
18. Harris, E.N., Gharavi, A.E., Hedge, U., Derue, G., Morgan, S.H., Englert, H., Chan, J.K.H., Asherson, R.A. and Hughes, G.R.V. 1985. Anticardiolipin antibodies in autoimmune thrombocytopenic purpura. *Br. J. Haematol.* **59**: 231–234.
19. Huisman, J.G. 1986. Immunoblotting: An emerging technique in immunohematology. *Vox Sang.* **50**: 129–136.
20. Kaplan, C., Champeix, P., Blanchard, D., Muller, J.Y. and Carton, J.P. 1987. Platelet antibodies in systemic lupus erythematosus. *Br. J. Haematol.* **67**: 89–93.
21. Karpatkin, S. 1985. Autoimmune thrombocytopenic purpura. *Semin. Hematol.* **22**: 260–288.
22. Kelton, J.G. and Gibbons, S. 1982. Autoimmune platelet destruction: Idiopathic thrombocytopenic purpura. *Semin. Thromb. Hemost.* **8**: 83–104.
23. Kelton, J.G., Powers, P.J. and Carter, C.J. 1982. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. *Blood* **60**: 1050–1053.
24. Kelton, J.G., Denomne, G., Lucarelli, A., Gravy, J., Powers, P. and Carter, C. 1985. Comparison of the measurement of surface or total platelet-associated IgG in the diagnosis of immune thrombocytopenia. *Am. J. Hematol.* **18**: 1–5.
25. Kelton, J.G., Murphy, W.G., Lucarelli, A., Garvey-Williams, J., Santos, A., Meyer, R. and Powers, P. 1989. A prospective comparison of four techniques for measuring platelet-associated IgG. *Br. J. Haematol.* **71**: 97–105.
26. van Leeuwen, E.F., van der Ven, J.Th.M., Engelfriet, C.P. and von dem Borne, A.E.G. 1982. Specificity of autoantibodies in autoimmune thrombocytopenia. *Blood* **59**: 23–26.
27. Lynch, D.M. and Howe, S.E. 1986. Antigenic determinants in idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **63**: 301–308.
28. Mason, D. and McMillan, R. 1984. Platelet antigens in chronic idiopathic thrombocytopenic purpura. *Br. J. Haematol.* **56**: 529–534.
29. McMillan, R. 1981. Chronic idiopathic thrombocytopenic purpura. *N. Engl. J. Med.* **304**: 1135–1147.
30. McMillan, R., Tani, P., Berchtold, P. and Millard, F. 1987. Detection of autoantibodies and alloantibodies against platelet-associated antigens. *Thromb. Hae-*

- most. 58: 530.
31. **Niessner, H., Clemetson, K.J., Panzer, S., Mueller-Eckhardt, C., Santoso, S. and Bettelheim, P.** 1986. Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies, *Blood* 68: 571–576.
  32. **Nomura, T.** 1984. Idiopathic thrombocytopenic purpura subcommittee report. p. 26–29. In Proc. Idiopathic Hematopoietic Disorders, Ministry of Welfare Cooperation Works. Tokyo. (In Japanese)
  33. **Pfueller, S.L. and David, R.** 1986. Liberation of surface and internal platelet-associated IgG (PA-IgG) during platelet activation. *Br. J. Haematol.* 63: 785–794.
  34. **Rosenfeld, S.I., Looney, R.J., Leddy, J.P., Phipps, D.C., Abraham, G.N. and Anderson, C.L.** 1985. Identification as a 40,000-molecular-weight membrane protein shared by monocytes, *J. Clin. Invest.* 76: 2317–2322.
  35. **Schiffer, C.A. and Young, V.** 1983. Detection of antibodies using a micro-enzyme-linked immunosolubent assay (ELISA). *Blood* 61: 311–317.
  36. **Steiner, M. and Lüscher, E.F.** 1986. Identification of the immunoglobulin G receptor of human platelets. *J. Biol. Chem.* 261: 7230–7235.
  37. **Stricker, R.B., Abrams, D.I., Corash, L. and Shuman, M.A.** 1985. Target platelet antigen in homosexual men with immune thrombocytopenia. *N. Engl. J. Med.* 313: 1375–1380.
  38. **Stricker, R.B., Reyes, P.T., Corash, L. and Schumann, M.A.** 1987. Evidence that a 210,000-molecular-weight glycoprotein (GP 210) serves as a platelet Fc receptor. *J. Clin. Invest.* 79: 1589–1594.
  39. **Szatkowski, N.S., Kunicki, J.J. and Aster, R.H.** 1986. Identification of glycoprotein Ib as a target for auto-antibody in idiopathic (autoimmune) thrombocytopenic purpura. *Blood* 67: 310–315.
  40. **Takamatsu, Y.** 1986. Significance of platelet-associated IgG (PA-IgG) in patients with chronic idiopathic thrombocytopenic purpura (chronic ITP). I. Detection of platelet-associated IgG by rocket immunoelectrophoresis. *Hiroshima Med. J.* 34: 1–14. (In Japanese).
  41. **Takamatsu, Y.** 1986. Significance of platelet-associated IgG (PA-IgG) in patients with chronic idiopathic thrombocytopenic purpura (chronic ITP). II. Clinical study on PA-IgG in patients with chronic ITP. *Hiroshima Med. J.* 34: 15–37. (In Japanese).
  42. **Tsubakio, T., Kurata, Y., Yonezawa, T. and Kitani, T.** 1981. Quantification of platelet-associated IgG with competitive solidphase enzyme immunoassay. *Acta Haematol.* 66: 251–256.
  43. **van Vliet, H.H.D.M., Kappers-Klunne, M.C. and Abels, J.** 1986. Pseudothrombocytopenia: a cold autoantibody against platelet glycoprotein GP IIb. *Br. J. Haematol.* 62: 501–511.
  44. **van Vliet, H.H.D.M., Kappers-Klunne, M.C., van der Hel, J.W.B. and Abels, J.** 1987. Antibodies against glycosphingolipids in sera of patients with idiopathic thrombocytopenic purpura. *Br. J. Haematol.* 67: 103–108.
  45. **Wood, V.L., Oh, E.H., Mason, D. and McMillan, R.** 1984. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood* 63: 368–375.
  46. **Wood, V.L., Kurata, Y., Montgomery, R.R., Tani, P., Mason, D., Oh, E.H. and McMillan, R.** 1984. Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. *Blood* 64: 156–160.