Mean platelet size related to glycoprotein-specific autoantibodies and platelet-associated IgG

K. JAVELA, R. KEKOMÄKI

OnlineOpen: This article is available free online at www.blackwell-synergy.com

Finnish Red Cross Blood Service, Helsinki, Finland

Correspondence:
Kaija Javela, Finnish Red Cross, Blood Service, Kivihaantie 7, FIN-00310, Helsinki, Finland.
Tel.: + 358 9 5801291;
Fax: +358 9 5801484;
E-mail: kaija.javela@bts.redcross.fi

SUMMARY

Recent evidence suggests that platelet-associated glycoprotein-specific (GP) antibodies represent true positive autoantibodies and can therefore be taken as the gold standard. Earlier tests, which aimed at detecting platelet-associated IgG (PA-IgG), might have been hampered, e.g. by the variation of platelet size in thrombocytopenic patients. In this study, 206 samples with increased PA-IgG from consecutive thrombocytopenic patients were tested further for GP-specific antibodies with a monoclonal antibody immobilized platelet antigen test (MAIPA) using a combination of a GP IIbIIIa-specific and a GP IbIX-specific antibody for immobilization or, in a separate assay, GP V-specific antibody. Mean platelet size was recorded as forward scatter (FSC) of platelets in flow cytometric analysis of PA-IgG. GP-specific antibodies were detected in 49 (24%) of the 206 patient samples. Their presence correlated well with increased PA-IgG ($R = 0.769$). The mean platelet size and mean fluorescence intensity (MFI) of PA-IgG were both significantly increased in patients compared with healthy controls ($n = 112$; $P < 0.0001$). Notably, PA-IgG was associated with platelet size within the platelet population of both healthy controls and patients ($R = 0.999$). Further, the probability of GP IIbIIIa and/or IbIX and GP V-specific PA-IgG tended to increase with the mean platelet size of the patients ($P = 0.045$). In conclusion, large platelets bound more IgG than platelets of normal size, which may explain at least in part the reported low specificity of total PA-IgG measurement. As the PA-IgG displays low specificity compared with the gold standard, its use as such may be abandoned and replaced by tests for platelet-associated GP-specific autoantibodies.

INTRODUCTION

There is a clinical need for improved non-invasive means to diagnose and predict the course of auto- immune thrombocytopenia (ITP). As the cause of elevated platelet-associated IgG (PA-IgG) in multiple thrombocytopenic states is still unclear, such tests have been considered unnecessary and even

inappropriate for establishing the diagnosis of TP (Mueller-Eckhardt *et al.*, 1980; George *et al.*, 1996). A part of the problem could reflect the variation of platelet size in thrombocytopenic patients (Illes *et al.*, 1987; Holme *et al.*, 1988b; Jackson & Carter, 1993).

Forward scatter signal distributions of platelets (FSC) in flow cytometry have been presented as fairly comparable with platelet size distributions as determined by hematology cell counter (Holme *et al.*, 1988b). Although flow cytometry has been applied for the detection of PA-IgG (Holme *et al.*, 1988a; Holme *et al.*, 1988b; Kokawa, Nomura & Yasunaga, 1991; Ault & Mitchell, 1994; Leytin *et al.*, 1996; Hagenstrom *et al.*, 2000), little attention has been paid to platelet size in the same measurement (Holme *et al.*, 1988b).

Glycoprotein (GP)-specificity of platelet-associated autoantibodies can be determined by antigen-specific assays, such as monoclonal antibody-specific immobilization of platelet antigen assay (MAIPA; Kiefel *et al.*, 1987), and radioactive immunobead assay (McMillan *et al.*, 1987). Tomer and collaborators reported that the presence of ITP was highly probable (>90%) if the antigen-specific assay was positive (Tomer, Koziol & McMillan, 2005). However, a negative assay does not rule out the diagnosis (McMillan, 1995; Tomer, Koziol & McMillan, 2005). Antigen-specific assays may be also useful in other thrombocytopenic patients than ITP (McMillan, 2005) and may be taken as the gold standard of platelet autoantibody assays.

The aims of the present study were (i) to characterize the normal reference ranges for PA-IgG and platelet size of a PA-IgG assay where results were obtained by flow cytometry, (ii) to study the correlation of platelet size with PA-IgG and (iii) to detect GP-specific autoantibodies with an independent assay in the patients with increased PA-IgG and (iv) to study whether the GP-specific autoantibodies were present more frequently in patients with platelets larger than normal.

MATERIALS AND METHODS

Patient samples

Samples from 854 thrombocytopenic patients were screened for PA-IgG. 206 samples with increased PA-IgG [mean fluorescence intensity (MFI) cut-off value above 300 as determined by mean+2s of healthy controls] formed the study population tested for GP-specific autoantibodies (Figure 1). Amongst other data, the lowest (nadir) and the actual platelet count were requested in the sample referrals. Although most referrals included suspected ITP, the clinical condition was multifactorial in most of cases. Thus, these patients were background patient material of this study. The focus, however, was on platelet size and GP-specific PA-IgG.

Healthy controls

Samples from 112 controls were obtained from healthy volunteer blood donors and handled similarly
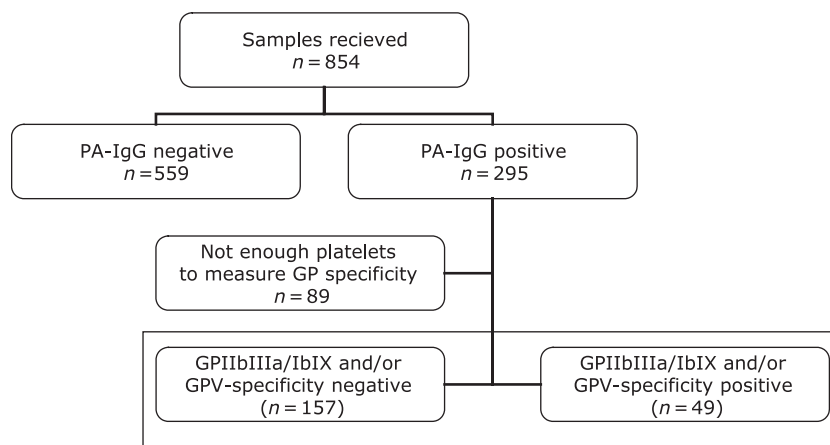

Figure 1.   Distribution of consequent patient samples according to screening for platelet-associated IgG (PA-IgG) and results of further determination of glycoprotein (GP)-specificity of PA-IgG.

to the patient samples. One sample was included in each run (see Controls).

Measurement of PA-IgG

Venous blood was collected in three 10 ml EDTA tubes (Venoject, Terumo 288267 or Vacutainer, Becton Dickinson 368457; Becton Dickinson, Franklin Lakes, NJ, USA), which were transported at room temperature. The samples were handled within 24 h.

PA-IgG analysis was performed as a screening method as described earlier (dem Borne *et al.*, 1978; Ault & Mitchell, 1994; Joutsi & Kekomaki, 1997). The analyses were performed using a FACScan flow cytometer and Cell Quest program, version 3.1f (Becton Dickinson, Mountain View, CA, USA). To enable analysis of thrombocytopenic samples, no more than 5000 cells were investigated from each sample. If necessary, the gate was adjusted daily according to the healthy control sample. The mean FSC and MFI of PA-IgG were recorded.

To analyze if platelets of different size in a single sample had different amounts of PA-IgG, the platelet scatter in FSC/SSC plot was divided into five consecutive gates (R1–R5). Altogether, 54 randomly selected samples were analyzed for PA-IgG using these gates (samples from patients with large, $n = 27$, with normal, $n = 22$, and with small platelets, $n = 5$). R3 was set to include 50% of events and both R2 and R4 20%. R1 and R5 were set to include events outside R2–R4 (Figure 2).

Controls

The preanalytical factors were covered by the use of controls, which were handled strictly in the same way as patient samples to minimize the influence of, e.g. whole blood storage prior to preparation (Hagenstrom *et al.*, 2000), EDTA (Lucas & Holburn, 1987; Kuijpers *et al.*, 1992; O'Malley *et al.*, 1996; Nishioka *et al.*, 2002), and fixation with formaldehyde (De Caterina *et al.*, 2002). The influence of preventive maintenance of flow cytometry on the precision of the assay was minimized by analyzing the controls and at least 10 patient samples before and after maintenance. The parameter settings were adjusted if there was a systematic difference of >3% (three times during 5 years).
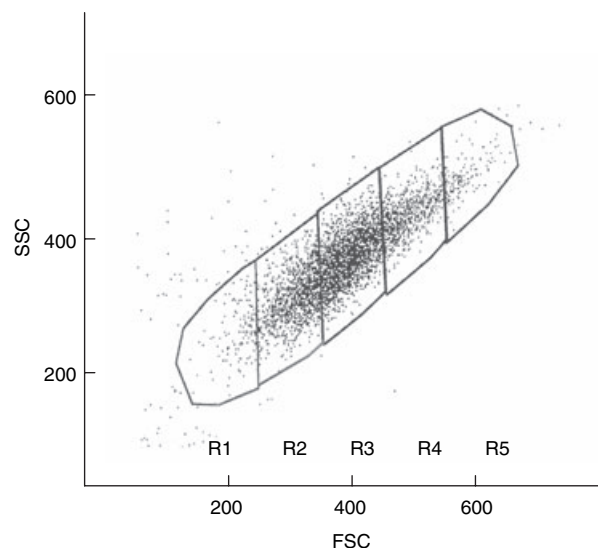

Figure 2. Platelet populations gated according to platelet size (forward scatter, FSC). Five regions of platelets from a healthy control sample in FSC/SSC (side scatter) dot plot were set: R3 to include 50% of events, both R2 and R4 20%, and R1 and R5 events left outside R2–R4.

A sample from a random healthy donor was used as a control to check all steps of analysis, and to follow the stability of the cut-off level. A strong positive control was used to monitor the linearity and stability of the measurement at high levels of PA-IgG. It controlled the analysis from the step of labeling of the platelets to the measurement. The control was prepared by incubating a pooled platelet preparation from six blood group O donors with 1 : 2 diluted serum containing strong anti-HPA-1a antibodies. The dilution level of anti-HPA-1a serum was chosen so that the PA-IgG result of the control would be as high as possible but still within the linear region of the fluorescence (data not shown). The same batch was used for up to 1 year.

A cut-off (positive) control was used to monitor the linearity and stability of the measurement near the positive (cut-off) level. The control was prepared by incubating a pooled platelet preparation from six blood group O donors with 1 : 64 dilution of the same serum as for the strong positive control. The same batch was used for up to 1 year. A new batch of all controls in the same series was compared to the previous batch for three to five times to establish new target values. Only one control was changed at a time.

GP-specific PA-IgG by direct MAIPA

GP-specific PA-IgG was measured by direct MAIPA as originally described (Kiefel *et al.*, 1987) and recently by us (Joutsi-Korhonen *et al.*, 2001). For measurement of GP IIbIIIa and/or IbIX specific antibodies, 25 µl of platelet suspension (1000×10^9 cells/l) was incubated for 30 min at 37 °C with 0.2 µg of two monoclonal antibodies in a single tube, one specific for the GP Ibα (AP1) and the other specific for the GP IIb-IIIa (AP2; both generous gifts from Doctors Kunicki and Montgomery, Blood Center of Southeastern Wisconsin, Milwaukee, WI, USA). In the separate measurement of GP V-specific antibodies, 80 µl of thawed platelet suspension (1000×10^9 cells/l) was incubated with 0.2 µg of monoclonal antibody specific for GP V (SW16, CLB, Netherlands). The secondary antibody was an affinity-purified goat antihuman IgG, Fc-specific, peroxidase labeled (Jackson 109-035-098; Suffolk, UK). The absorbances were measured at 492 nm with a spectrophotometer applying special software (Multiscan EX, Thermo Electron Corporation, Vantaa, Finland). To enable inter- and intralaboratory comparisons, the absorbance values of patient samples were divided by the absorbance of a negative control in each test series to get the ratio value, as recommended in the literature (Berchtold *et al.*, 1997). A sample was considered to have increased GP-specific PA-IgG when the ratio was ≥2.0.

Controls for the MAIPA

Platelets with GP IIbIIIa-specific PA-IgG (anti-HPA-1a sensitized platelets) and platelets with increased GP V-specific PA-IgG (Joutsi-Korhonen *et al.*, 2001) were used as controls to monitor the measuring range of the assay.

Comparability of platelet size measurements by two cell counters and flow cytometry

In preliminary experiments, the platelet count and mean platelet volume (MPV) of 52 consecutive patient samples were determined both with an electric cell counter applying the impedance method (Medonic, Solna, Sweden) and another cell counter applying the hydrodynamic focusing method (Sysmex XT-2000i, Sysmex Corporation, Kobe, Japan). The results corre-

lated well ($n = 52$; $R = 0.954$, data not shown). The impedance method spared the samples of thrombocytopenic patients. Platelet size (MPV, range 10–16 fl; impedance method) correlated well with the mean of FSC (range 268–552) obtained by flow cytometry ($n = 32$; $R = 0.834$; data not shown). The reference range of healthy control samples ($n = 40$) was 7–10 fl.

Quality assurance of the methods

Westgard multirule quality control rules 1_{3s}, 2_{2s}, and 4_{1s} were applied to control results to detect random and systematic errors (Westgard *et al.*, 1981).

Precision, cut-off values and reference intervals of the PA-IgG assay

The precision of the PA-IgG method measured as intra-assay and inter-assay repeatability of control samples varied at low level from 1.9% to 4.5% and at high level from 0.4% to 1.6% (Table 1). The intra-assay and inter-assay repeatability of the GP IIbIIIa and/or IbIX- and GP V-specific determinations of PA-IgG were 5.8% and 12%, which is typical of ELISA methods.

Long-term precision was evaluated by monitoring the annual cut-off value of PA-IgG, which was calculated from the results of donor samples (mean+2s; 300–309), and monitoring the stability of the reference interval for the FSC of the healthy control population (mean ± 2s; 310–376; Table 2).

The reference intervals of the FSC and cut-off-values of PA-IgG did not differ statistically significantly during the five consecutive years ($P > 0.999$, Table 2). The results indicate good long-term stability. Patient means were analyzed to detect any long-term drift in PA-IgG measurement (Bull *et al.*, 1974; Table 2).

Statistics

Microsoft® Excel 2003 (Microsoft Corporation) and StatsDirect (version 1.8.10) were used for statistical analysis (mean, standard deviation, SD, coefficient of variation, *CV*, and skewness of distribution). The correlations between methods, R^2, and the significance of R were calculated with multiple linear regression tests. The Mann–Whitney test was used to calculate *P*-values. The κ^2-test was used to study the significance of differences in GP IIbIIIa and/or IbIX and GP

Table 1. Precision of platelet-associated IgG (PA-IgG) and glycoprotein (GP)-specific assays

| Control level | PA-IgG assay | | | | GP-specific assays | | | |
| | Intra-assay CV | | Inter-assay CV | | Intra-assay CV | | Inter-assay CV | |
	FSC	PA-IgG (MFI)	FSC	PA-IgG (MFI)	GPIIbIIIa/IbIX	GPV	GPIIbIIIa/IbIX	GPV
Low	1.9	2.3	3.4	4.5	2.5	2.1	5.3	4.5
High	0.4	0.7	0.6	1.6	5.8	5.1	12.3	11.5

Values are expressed as precentage (%).
FSC, forward scatter; PA-IgG, platelet associated IgG; MFI, mean fluorescence intensity; GP IIbIIIa and/or IbIX and GP V, glycoprotein specificity of autoantibodies.

Table 2. Reference intervals of FSC and cut-off values of platelet-associated IgG (PA-IgG)

| Year | n | Healthy controls | | | | Patients | | | |
| | | FSC | PA-IgG (MFI) | | | FSC | | PA-IgG (MFI) | |
		Mean ± 2SD*	Mean + 2SD†	n		Mean	SD	Mean	SD
1	108	318–385	302	1012		389	29.9	291	98.5
2	102	320–386	309	965		374	27.5	290	75.3
3	111	306–366	301	866		377	28.0	294	99.2
4	108	304–387	309	886		374	34.7	333	78.6
5	112	309–376	300	854		369	30.0	316	69.6

*Annual reference intervals (mean ± 2SD) for forward scatter signal distributions of platelets (FSC).
†95% cut-off values for PA-IgG (mean+2SD) from healthy control results. Annual control means did not differ statistically from each other ($P > 0.999$) whereas patient means were higher than the control means ($P < 0.0001$).

V results grouped according to platelet size. *P*-values below 0.05 were considered statistically significant.

RESULTS

Platelet size (FSC) related to PA-IgG

In all screened patients, there was more PA-IgG in samples of platelets from patients than of platelets from healthy controls (316 ± 69.6 and 219 ± 40.7, respectively; $P < 0.0001$; Figure 3a).

The mean FSC of the patient population was significantly higher than that of the healthy control population (369 ± 30 and 342 ± 17, respectively; $P < 0.0001$; Figure 3b). Thirty-four percent of patient samples were on the upper side of the reference interval (FSC > 376) and 0.6% below (FSC < 306).

Of the 854 patient samples, 295 had increased PA-IgG (MFI > 300; Figure 1). PA-IgG was directly associated with platelet size within gated platelet populations of both control and patient samples (Figure 4; $R = 0.999$).

GP-specificity of PA-IgG

GP IIbIIIa and/or IbIX-specific PA-IgG was detectable in 44 samples (21%) and GP V-specific PA-IgG in 25 of 206 samples (12%). Only low level of GP V-specific PA-IgG was found in five samples without GP IIbIIIa and/or IbIX-specificity. GP IIbIIIa and/or IbIX-specific PA-IgG was directly associated with GP V-specific PA-IgG ($R = 0.374$, data not shown). Altogether GP IIbIIIa and/or IbIX and/or GP V-specific PA-IgG were detected in 49 of 206 samples (24%; Figure 1).
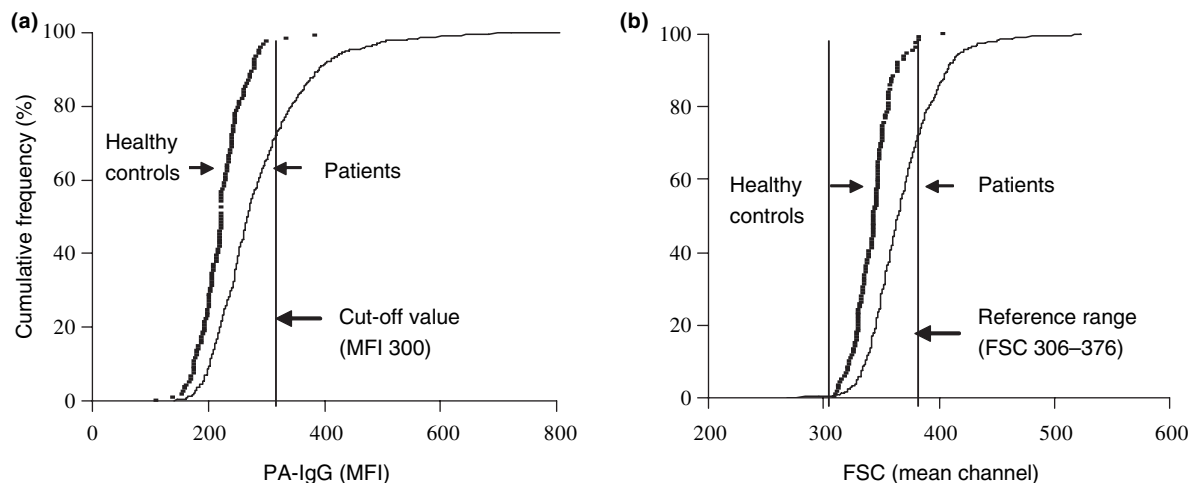
Figure 3. Cumulative frequency distributions of platelet-associated IgG (PA-IgG; a) and forward scatter signal distribution of platelets (FSC; b) in healthy controls ($n = 112$) and in all screened patients ($n = 854$; $P < 0.0001$).
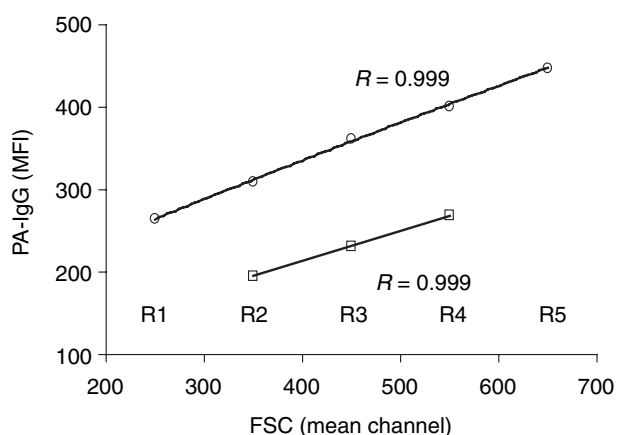

Figure 4. Association of platelet-associated IgG (PA-IgG; MFI) with platelet size within the platelet populations of study subjects (grouped according to gated regions, see Figure 2; patients (○); $n = 54$, and healthy controls (□); $n = 28$).

PA-IgG was directly associated with the presence of GP IIbIIIa and/or IbIX, and/or GP V-specific PA-IgG (Figure 5a, $R = 0.769$). When compared with healthy control samples, the cumulative frequency distributions of FSC were significantly higher in patients where GP-specific PA-IgG could be studied ($P < 0.0001$). However, platelets were only slightly larger in patients with GP-specific PA-IgG than in patients without ($n = 49$ and $n = 157$, $P = 0.048$; Figure 5b).

GP IIbIIIa and/or IbIX, and/or GP V-specific PA-IgG were more frequently detected in patient samples with larger platelets than in samples with normal sized platelets ($P = 0.045$). Thirty two percent of patients with larger platelets had GP-specific antibodies, compared with 19% of patients with platelets of normal size.

Platelet autoantibodies and platelet count

The historical platelet nadir recorded in the referrals of the 27 of 44 patients with GP-specific PA-IgG displayed negative correlation with the level of GP IIbIIIa and/or IbIX-specific PA-IgG ($R = -0.544$, data not shown).

DISCUSSION

Monitoring measures of platelet size, such as mean platelet volume (Illes *et al.*, 1987; George *et al.*, 1996; Rajantie *et al.*, 2004), size deviation width (PDW) and platelet large cell ratio (P-LCR; Kaito *et al.*, 2005), as well as proportion of immature platelet fraction (Briggs *et al.*, 2004) have been suggested to aid in the diagnosis of thrombocytopenia.

Until now, relatively little attention has been paid to evaluating of platelet size in association with platelet antibody studies (Holme *et al.*, 1988b; Kokawa, Nomura & Yasunaga, 1991). In this study, platelet size
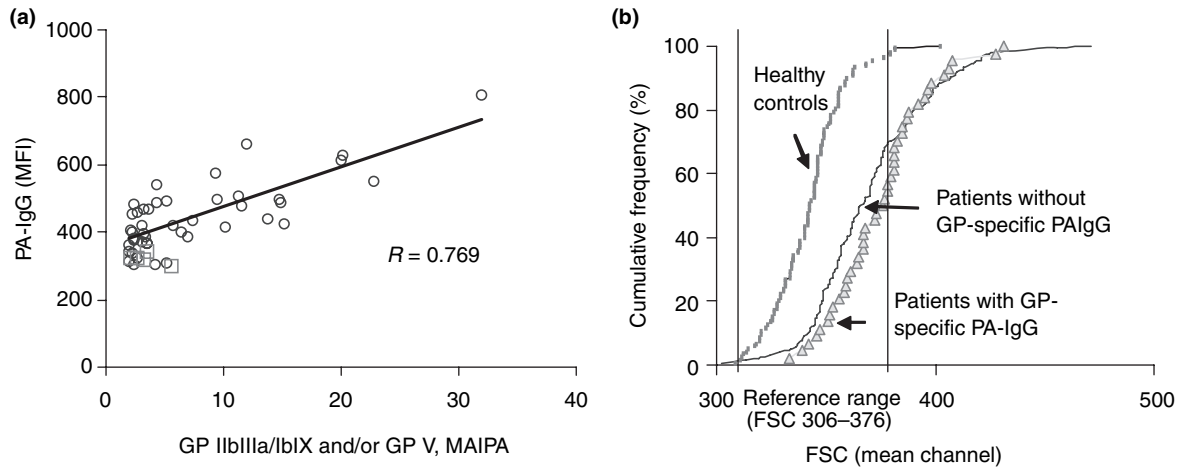
Figure 5. The association of GP IIbIIIa/IbIX-specific antibodies and PA-IgG ($n = 49$; (○). In addition, low level of GP V-specific PA-IgG was found in five samples without GP IIbIIIa and/or IbIX-specificity (□; a). Cumulative frequency distributions of FSC were significantly higher in patients where GP-specific PA-IgG could be studied ($n = 49$; $P < 0.0001$). Platelets were only slightly larger in patients with GP-specific PA-IgG than in patients without ($n = 49$ and 157, respectively; $P = 0.048$; b).

(FSC) as well as PA-IgG were statistically higher in patients than in healthy controls on the analysis of PA-IgG. Furthermore, PA-IgG correlated directly with the FSC within platelet populations of both healthy donors and patients. Thus, the larger the platelet was, the more IgG was bound by the cell. As the PA-IgG is indeed related to FSC, it suggests the possibility of estimating the amount of IgG bound per unit platelet surface area (Leytin *et al.*, 1996). The diagnostic value of platelet size distributions of a sample should be tested in detection of autoantibodies in patients with definitive ITP and other thrombocytopenic states.

The reference ranges of both FSC and PA-IgG in PA-IgG assay remained within narrow limits. To obtain reproducible results for this analysis, it was necessary to perform laboratory measurements in a strictly controlled way. In this context, rules originally developed for quantitative measurements were applied and seemed to work well (Bull *et al.*, 1974; Westgard *et al.*, 1981; Cembrowski & Westgard, 1985; Lott *et al.*, 1996). This was especially important because international quality assurance for the measurement of platelet autoantibodies is still not available.

In the present study, PA-IgG showed strong direct association with GP IIbIIIa and/or IbIX and/or GP V-specific PA-IgG, which was taken as the gold standard for platelet autoantibody assays. GP-specific auto-

antibodies have been detected in varying proportions in autoimmune thrombocytopenia (McMillan, Wang & Tani, 2003). Brighton *et al.* (1996) reported a positivity rate of 49% in 66 ITP-patients and in 6/15 patients with secondary immune TP. Also, 22% of patients with initial diagnosis of non-immune TP were reported positive. McMillan, Wang & Tani (2003) studied 282 chronic ITP patients, and 55.4% of them had GP IIbIIIa and/or IbIX-specific PA-IgG. In the present study, GP IIbIIIa/IbIX and/or GP V-specific autoantibodies were observed in a quarter of patients who had sufficient platelets to measure GP-specificity. The low sensitivities of GP-specific PA-IgG may vary according to the selection of the patient population. In a strictly diagnosed ITP with extensive exclusion of other causes of thrombocytopenia, higher sensitivity of GP-specific antibody assay would be expected (McMillan, Wang & Tani, 2003). Also, the selection of the cut-off value has an influence on the assay sensitivity. The proportion of patients with GP-specific antibodies would have been larger if the cut-off value of +3s (MFI 340) had been used instead of +2s (MFI 300) for PA-IgG. Although GP-specific antibodies were not tested in the patient population without PA-IgG, the probability of finding GP-specific autoantibodies in such patients would have remained low. The previous results from the same laboratory indicated that when

applying two standard deviations as the cut-off value only 2% of patients without increased PA-IgG had slightly increased GP-specific antibodies (Joutsi & Kekomaki, 1997). A suboptimal combination of monoclonal antibodies applied in the detection of GP-specificity might also have contributed to the proportion of samples with GP-specific antibodies.

In the present study, the frequency of GP IIbIIIa and/or IbIX, and/or GP V-specific PA-IgG was statistically higher in patients with larger platelets although the platelets were still within the reference range (FSC). Interestingly, Kokawa, Nomura & Yasunaga (1991) reported larger platelet volume in ITP patients without GP IIbIIIa-specific antibodies than in those with GP IIbIIIa-specific antibodies. Their separate analyses of GP IIbIIIa and IbIX specific antibodies may explain the disparity in results.

Glycoprotein IIbIIIa has been shown to carry the most common target antigens in ITP (Beardsley et al., 1984; McMillan et al., 1987; Kekomaki et al., 1991). In addition, GP IbIX is a prominent target antigen (Kiefel et al., 1991). In this study, GP IIbIIIa-specific PA-IgG was measured together with GP IbIX PA-IgG. The significance of GPV-specific PA-IgG remained unclear. Interestingly, antigen-specific assays have been reported to demonstrate high specificity in the diagnosis of ITP (McMillan, 2005; Tomer, Koziol & McMillan, 2005).

In conclusion, it was possible to achieve reproducible results in the PA-IgG and GP-specific PA-IgG assays. The high frequency of increased PA-IgG by flow cytometry suggested low specificity as GP-specificity of the antibodies could be demonstrated relatively rarely. Thus every attempt should be made to spare all platelets of the sample for direct measurement of GP-specific PA-IgG. Even if platelet size was measured separately by a hematology analyzer, the results should be analyzed together with the platelet antibody assay. When interpreting the size of platelets, other disorders in addition to ITP that can cause macrothrombocytopenia with shortened platelet survival time have to be kept in mind, e.g. Bernard-Soulier disease (Koskela et al., 1999). Prospective studies are indicated to test the diagnostic and predictive value of tests for GP-specific antibodies and platelet size in TP patients.

ACKNOWLEDGEMENTS

REFERENCES

Ault K.A. & Mitchell J. (1994) Analysis of platelets by flow cytometry. Methods in Cell Biology 42(Pt B), 275–294.

Beardsley D.S., Spiegel J.E., Jacobs M.M., Handin R.I. & Lux S.E. (1984) Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. Journal of Clinical Investigation 74, 1701–1707.

Berchtold P., Muller D., Beardsley D., Fujisawa K., Kaplan C., Kekomaki R., Lipp E., Morell-Kopp M.C., Kiefel V., McMillan R., dem Borne A.E. & Imbach P. (1997) International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies. British Journal of Haematology 96, 477–483.

dem Borne A.E., Verheugt F.W., Oosterhof F., von Riesz E., de la Riviere A.B. & Engelfriet C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. British Journal of Haematology 39, 195–207.

Briggs C., Kunka S., Hart D., Oguni S. & Machin S.J. (2004) Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. British Journal of Haematology 126, 93–99.

Brighton T.A., Evans S., Castaldi P.A., Chesterman C.N. & Chong B.H. (1996) Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood 88, 194–201.

Bull B.S., Elashoff R.M., Heilbron D.C. & Couperus J. (1974) A study of various estimators for the derivation of quality control procedures from patient erythrocyte indices. American Journal of Clinical Pathology 61, 473–481.

Cembrowski G.S. & Westgard J.O. (1985) Quality control of multichannel hematology analyzers: evaluation of Bull's algorithm. American Journal of Clinical Pathology 83, 337–345.

De Caterina M., Grimaldi E., Ungaro B., Fratellanza G., Varriale V., Ciarnelli M. & Scopacasa F. (2002) Effect of paraformaldehyde on platelet size and on measurement of surface IgG. Platelets 13, 207–212.

George J.N., Woolf S.H., Raskob G.E., Wasser J.S., Aledort L.M., Ballem P.J., Blanchette V.S., Bussel J.B., Cines D.B., Kelton J.G., Lichtin A.E., McMillan R., Okerbloom J.A., Regan D.H. & Warrier I. (1996) Idiopathic thrombocytopenic purpura: a practice guideline developed

by explicit methods for the American Society of Hematology. Blood 88, 3–40.

Hagenstrom H., Schlenke P., Hennig H., Kirchner H. & Kluter H. (2000) Quantification of platelet-associated IgG for differential diagnosis of patients with thrombocytopenia. Thrombosis and Haemostasis 84, 779–783.

Holme S., Heaton A., Konchuba A. & Hartman P. (1988a) Light scatter and total protein signal distribution of platelets by flow cytometry as parameters of size. Journal of Laboratory and Clinical Medicine 112, 223–231.

Holme S., Heaton A., Kunchuba A. & Hartman P. (1988b) Increased levels of platelet associated IgG in patients with thrombocytopenia are not confined to any particular size class of platelets. British Journal of Haematology 68, 431–436.

Illes I., Pfueller S.L., Hussein S., Chesterman C.N. & Martin J.F. (1987) Platelets in idiopathic thrombocytopenic purpura are increased in size but are of normal density. British Journal of Haematology 67, 173–176.

Jackson S.R. & Carter J.M. (1993) Platelet volume: laboratory measurement and clinical application. Blood Reviews 7, 104–113.

Joutsi L. & Kekomaki R. (1997) Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG. British Journal of Haematology 96, 204–209.

Joutsi-Korhonen L., Javela K., Hormila P. & Kekomaki R. (2001) Glycoprotein V-specific platelet-associated antibodies in thrombocytopenic patients. Clinical and Laboratory Haematology 23, 307–312.

Kaito K., Otsubo H., Usui N., Yoshida M., Tanno J., Kurihara E., Matsumoto K., Hirata R., Domitsu K. & Kobayashi M. (2005) Platelet size deviation width, platelet large cell ratio, and mean platelet volume have sufficient sensitivity and specificity in the diagnosis of immune thrombocytopenia. British Journal of Haematology 128, 698–702.

Kekomaki R., Dawson B., McFarland J. & Kunicki T.J. (1991) Localization of human platelet autoantigens to the cysteine-rich region of glycoprotein IIIa. Journal of Clinical Investigation 88, 847–854.

Kiefel V., Santoso S., Weisheit M. & Mueller-Eckhardt C. (1987) Monoclonal antibody–specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood 70, 1722–1726.

Kiefel V., Santoso S., Kaufmann E. & Mueller-Eckhardt C. (1991) Autoantibodies against platelet glycoprotein Ib/IX: a frequent finding in autoimmune thrombocytopenic purpura. British Journal of Haematology 79, 256–262.

Kokawa T., Nomura S. & Yasunaga K. (1991) Relationship between platelet volume and anti-platelet autoantibodies in idiopathic thrombocytopenic purpura. European Journal of Haematology 47, 104–108.

Koskela S., Javela K., Jouppila J., Juvonen E., Nyblom O., Partanen J. & Kekomaki R. (1999) Variant Bernard-Soulier syndrome due to homozygous Asn45Ser mutation in the platelet glycoprotein (GP) IX in seven patients of five unrelated Finnish families. European Journal of Haematology 62, 256–264.

Kuijpers R.W., Ouwehand W.H., Peelen W., Michiels J.J., Engelfriet C.P. & dem Borne A.E. (1992) Thrombocytopenia due to platelet glycoprotein IIb/IIIa-reactive autoantibodies non-reactive with platelets from EDTA blood. Vox Sanguinis 63, 119–121.

Leytin V., Shapiro H., Novikov I. & Radnay J. (1996) Flow cytometric analysis of the platelet surface area and surface density of glycoprotein IIb-IIIa of unactivated human platelets of various sizes. Biochemical and Biophysical Research Communications 226, 94–100.

Lott J.A., Smith D.A., Mitchell L.C. & Moeschberger M.L. (1996) Use of medians and 'average of normals' of patients' data for assessment of long-term analytical stability. Clinical Chemistry 42, 888–892.

Lucas G.F. & Holburn A.M. (1987) The effect of anticoagulant on platelet associated IgG. British Journal of Haematology 65, 111–115.

McMillan R. (1995) Clinical role of antiplatelet antibody assays. Seminars in Thrombosis and Hemostasis 21, 37–45.

McMillan R. (2005) The role of antiplatelet autoantibody assays in the diagnosis of immune thrombocytopenic purpura. Current Hematology Reports 4, 160–165.

McMillan R., Tani P., Millard F., Berchtold P., Renshaw L. & Woods V.L. Jr (1987) Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. Blood 70, 1040–1045.

McMillan R., Wang L. & Tani P. (2003) Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). Journal of Thrombosis and Haemostasis 1, 485–491.

Mueller-Eckhardt C., Kayser W., Mersch-Baumert K., Mueller-Eckhardt G., Breidenbach M., Kugel H.G. & Graubner M. (1980) The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. British Journal of Haematology 46, 123–131.

Nishioka T., Yokota M., Tsuda I. & Tatsumi N. (2002) Flow cytometric analysis of platelet activation under calcium ion-chelating conditions. Clinical and Laboratory Haematology 24, 115–119.

O'Malley T., Ludlam C.A., Fox K.A. & Elton R.A. (1996) Measurement of platelet volume using a variety of different anticoagulant and antiplatelet mixtures. Blood Coagulation and Fibrinolysis 7, 431–436.

Rajantie J., Javela K., Joutsi-Korhonen L. & Kekomaki R. (2004) Chronic thrombocytopenia of childhood: use of non-invasive methods in clinical evaluation. European Journal of Haematology 72, 268–272.

Tomer A., Koziol J. & McMillan R. (2005) Autoimmune thrombocytopenia: flow cytometric determination of platelet-associated autoantibodies against platelet-specific receptors. Journal of Thrombosis and Haemostasis 3, 74–78.

Westgard J.O., Barry P.L., Hunt M.R. & Groth T. (1981) A multi-rule Shewhart chart for quality control in clinical chemistry. Clinical Chemistry 27, 493–501.