International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies

P. BERCHTOLD, 1 D. MÜLLER, 1 D. BEARDSLEY, 2 K. FUJISAWA, 3 C. KAPLAN, 4 R. KEKOMÄKI, 5 E. LIPP, 1 M. C. MORELL-KÖPF, 4 V. KIEFEL, 6 R. MCMILLAN, 7 A. E. G. KR. VON DEM BORNE 8 AND P. IMBACH 1

1 Department of Medicine, University Hospital, Bern and Basel, Switzerland, and the International Workshop Group from 2 New Haven, Connecticut, U.S.A., 3 Tokyo, Japan, 4 Paris, France, 5 Helsinki, Finland, 6 Giessen, Germany, 7 San Diego, California, U.S.A., and 8 Amsterdam, The Netherlands

Received 8 July 1996; accepted for publication 11 November 1996

Summary. Platelet-associated and plasma autoantibodies against platelet glycoproteins (GP) have been demonstrated in patients with autoimmune thrombocytopenia (AITP) using various methods. Eight laboratories in seven countries participated in this international study to evaluate the interlaboratory agreement using glycoprotein-specific immunoassays for these autoantibodies. The participating laboratories received blind samples of frozen washed platelets and plasma from 22 normal donors and 22 AITP patients. Platelet-associated and plasma autoantibodies against GPIIb–IIIa and GPIb–IX were measured by MAIPA, immunobead assay or modified antigen capture assay. Of the control samples, 96.0% and 97.2% of all results for platelet-associated and plasma autoantibodies to GPIIb–IIIa/GPIb–IX, respectively, were negative. The mean variation coefficient of the control samples of platelet-associated and plasma autoantibodies was 89.5% (range 11.1–272.9%) and 46.5% (range 21.0–78.0%), respectively. In 20/22 patient samples, platelet-associated autoantibodies to either glycoprotein were noted by at least two laboratories. The mean degree of agreement in these samples was 74.0%. There was a significant correlation in the individual antibody measurements between all laboratories (Kendall coefficient of concordance 0.60 and 0.38, P < 0.001; Spearman rank order test, range of correlation coefficient 52.3–94.0% and 42.2–85.0%, P < 0.05, for anti-GPIIb–IIIa and anti-GPIb–IX, respectively). In contrast, plasma autoantibodies to either glycoprotein were noted by at least two laboratories in only 13/22 patient samples. Moreover, the degree of agreement was poor (50.1%) and a significant correlation was noted between only six pairs of laboratories.

We conclude that methods used in this study yield good interlaboratory agreement in measuring platelet-associated autoantibodies against GPIIb–IIIa and GPIb–IX. In contrast, poor agreement was found in detecting plasma autoantibodies to the same glycoproteins.

Keywords: autoimmune thrombocytopenic purpura, autoantibody measurement, anti-GPIIb–IIIa, anti-GPIb–IX, glycoprotein-specific immunoassays.

Autoimmune thrombocytopenia (AITP) is characterized by increased platelet destruction due to antiplatelet antibodies that results in increased platelet clearance by the reticuloendothelial system (McMillan, 1981; Kelton & Gibbons, 1982). Several studies have demonstrated autoantibodies against platelet membrane glycoprotein (GP) IIb–IIIa and GPIb–IX in patients with AITP (Woods et al, 1984a, b; McMillan et al, 1987; Kiefel et al, 1987, 1991; Berchtold & Wenger, 1993). It has been shown that these autoantibodies are related to the activity of the disease and are not detected in normals or in non-thrombocytopenic patients (McMillan et al, 1987; Berchtold & Wenger, 1993). Therefore it has been postulated that antiglycoprotein autoantibodies play an important role in the pathogenesis of AITP (Berchtold & Wenger, 1993). These autoantibodies are detected in the majority of AITP patients as platelet-associated antibodies, whereas plasma autoantibodies are noted in a smaller number of patients (McMillan et al, 1987; Berchtold & Wenger, 1993). This difference may be important, since the specificity of antibodies bound to the platelet may differ from that of plasma antibodies and some of the latter are directed against antigens on the cytoplasmic portion of GPIIIa (Fujisawa et al, 1991, 1992).

Several glycoprotein-specific immunoassays, using monoclonal antibodies, suitable for the routine laboratory have
have been developed (McMillan et al., 1987; Kiefel et al., 1987). Given the pathogenetic importance of these autoantibodies and the potential diagnostic usefulness in AITP patients, we believe that it is necessary to compare these methods before their widespread use. We therefore designed a study to evaluate the interlaboratory agreement using antigen-specific tests capable of measuring platelet-associated as well as plasma autoantibodies against platelet GPIIb–IIIa and GPIb–IX. In 1993 a pilot study on 10 healthy controls and five patients with AITP revealed good agreement between the participating laboratories. In this paper we report the results of the first international study to compare methods used for detection of antiglycoprotein autoantibodies of patients with AITP.

MATERIALS AND METHODS

Preparation of samples. EDTA-anticoagulated blood from 22 AITP patients was collected and sent by overnight mail to Bern, Switzerland. The blood was immediately processed, and platelet and plasma samples were prepared by differential centrifugation. The platelets were washed six times with 0.05 M isotonic citrate buffer, resuspended and frozen in aliquots of 2 × 10^7 cells in 300 μl for later assay. Plasma samples were frozen in aliquots of 1 ml. All patients had been diagnosed as AITP (17 with primary AITP, two with AITP associated with systemic lupus erythematosus, one with Evans syndrome, one with Sjögren’s syndrome and one with vasculitis). At the time of diagnosis, each patient was thrombocytopenic and had no evidence for any other cause of thrombocytopenia (e.g. acute leukaemia, aplastic anaemia, etc.). At the time of blood sampling, 7/22 patients had recovered from thrombocytopenia due to immunosuppressive therapy. Each of the patients had detectable levels of autoantibody as demonstrated by the investigator who submitted the sample. Median age (range) and median platelet count (range) at the time of blood sampling was 33 years (8–74) and 99 × 10^9/l (14–843), respectively. Blood from 22 healthy adult blood donors was collected in Bern and normal platelet and plasma samples were prepared as described above.

Two sets of 44 blinded vials containing normal or patient platelets as well as two sets of 44 blinded vials containing normal or patient plasma were sent to each participating laboratory. The participants were selected from the pilot study if they were using one of the two antigen-specific methods listed below. All samples were shipped frozen. Two of the participating laboratories have previously studied the effect of freezing platelets on the detection of autoantibodies and there was no difference between fresh and frozen samples (data not shown). Two forms were provided to return the results of platelet-associated and plasma antibodies to GPIIb–IIIa and GPIb–IX as raw data, either as counts per minute (cpm) or optical density (OD). The participants were also requested to give a summary of their assay method. Patient antibody results are expressed as a binding ratio of the patient cpm/OD-value divided by the mean of the 22 normal samples. Patient samples with an antibody binding ratio above the mean + 3 SD of the controls found by at least two laboratories were considered positive.

Statistical methods. The variation coefficient of normal and patient samples was calculated as standard deviation x100 divided by the mean. Agreement between participating laboratories was calculated for each sample as percent negative and positive results for anti-GPIIb–IIIa/anti-GPIb–IX antibodies in normal controls and patient samples, respectively. Overall correlation of patient results between all laboratories was expressed as coefficient of concordance according to Kendall/Babington-Smith. Correlation of patient results between pairs of laboratories was calculated by Spearman rank order test.

Assays for anti-glycoprotein antibodies. Two different methods capable of measuring anti-GPIIb–IIIa and anti-GPIb–IX antibodies using murine monoclonal antibodies (MoAb) against these glycoproteins have been used in this study. The immunobead assay and the modified antigen capture assay are based on the same method using only different solid phases and detection systems (Table I).

MAIPA. Microtitre wells were coated with goat anti-mouse IgG by overnight incubation at 4°C and then washed and blocked with Tris-buffered saline (TBS). For measurement of platelet-associated autoantibodies, blinded platelet samples were incubated with monoclonal anti-GPIIb–IIIa or anti-GPIb–IX. To evaluate plasma antibodies, normal platelets were first incubated with the plasma sample, washed and then incubated with anti-glycoprotein MoAb. For assay, the platelets were then washed three times with isotonic saline and solubilised with 0.5% Triton X-100 in TBS. The platelet
lysates were then centrifuged for 30 min at 15 000 g. The supernatants were diluted in TBS and transferred to microtitre wells coated with goat anti-mouse IgG. After incubation for 90 min at 4°C, the wells were washed and reacted with horseradish peroxidase labelled or alkaline phosphatase labelled goat anti-human IgG for 120 min at 4°C for platelet-associated and plasma autoantibodies, respectively. After washing the wells, substrate solution (O-phenylenediamine and p-nitrophenylphosphate for platelet-associated and plasma autoantibodies, respectively) was added and absorbance was measured at 492 nm or 405 nm.

**Immunobead assay (IBA).** Immunobeads were prepared by incubating 1/4-inch polystyrene beads (Pierce Chemical Company, Rockford, Illinois) overnight with MoAb against either GPIIb–IIIa or GPIb–IX. After washing the beads with 2% BSA in PBS-Tween, unbound binding sites were blocked by incubating the beads with 2% bovine serum albumin (BSA) in PBS-Tween for 60 min. For assay, the blinded samples of washed platelets or normal platelets sensitized with the blinded plasma samples were solubilized in 1% Triton X-100. The solubilized platelets were added in triplicate to the wells and incubated for 1 h. After washing five times with PBS-Tween, bound anti-glycoprotein antibody was detected by consecutive incubation for 60 min with 2 μg/ml biotinylated monoclonal anti-human IgG (HB 43, American Type Culture Collection, Rockville, Md.).

**Modified antigen capture assay (MACA).** Microtitre wells were coated with MoAb either against GPIIb–IIIa or GPIb–IX for 2 h at room temperature. After washing five times with 0.05% Tween in PBS (PBS-Tween), unsaturated binding sites were blocked with 2% BSA in PBS-Tween. For assay, the blinded samples of washed platelets or normal platelets sensitized with the blinded plasma samples were solubilized in 1% Triton X-100. The solubilized platelets were added in triplicate to the wells and incubated for 1 h. After washing five times with PBS-Tween, bound anti-glycoprotein antibody was detected by radiolabelled monoclonal anti-human IgG (HB 43, American Type Culture Collection, Rockville, Md.).

**RESULTS**

Twenty-two blinded platelet and plasma samples of 22 healthy controls and AITP patients were distributed to each of eight participating laboratories. Results on platelet-associated and plasma antibodies to GPIIb–IIIa and GPIb–IX were obtained for all samples and from all participants.
### Table III.
Agreement and variation coefficient of platelet-associated autoantibody results in patient samples (S1–S60). Agreement was calculated as per cent positive results for the individual sample. Variation coefficients are expressed as per cent ratio of SD/mean of positive results (see Materials and Methods).

|       | S1  | S2  | S14 | S16 | S17 | S20 | S24 | S27 | S30 | S31 | S33 | S36 | S37 | S41 | S42 | S45 | S47 | S50 | S54 | S57 | S60 | Mean |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Anti-GPIIb–IIIA** |
| Agreement (%) | 63  | 100 | 50  | 100 | 63  | 38  | 63  | 100 | —   | 63  | 25  | 88  | 25  | 88  | 25  | 75  | 38  | —   | 88  | 100 | 66·9 |
| Variation (%)  | 81·8| 95·2| 56·8| 105·2| 87·1| 54·3| 127·2| 120·5| —   | 125·9| 54·7| 104·9| 92·3| 128·9| 95·3| 144·0| 129·6| —   | 112·2| 170·6| 104·8|
| **Anti-GPIb–IX** |
| Agreement (%) | 88  | 75  | 25  | 88  | 38  | 50  | 75  | 75  | —   | 75  | 50  | 25  | 88  | —   | 75  | —   | 38  | 25  | 25  | 25  | 55·3 |
| Variation (%)  | 116·4| 62·1| 132·8| 113·1| 89·8| 59·4| 123·8| 67·6 | —   | 144·1| 72·0| 86·9| 93·6| 75·2| —   | 45·8| 84·6| 101·8| 78·6| 91·0|

### Table IV.
Agreement and variation coefficient of plasma autoantibody results in patient samples (S1–S58). Agreement was calculated as per cent positive results for the individual sample. Variation coefficients are expressed as per cent ratio of SD/mean of positive results (see Materials and Methods).

|       | S1  | S3  | S6  | S8  | S13 | S14 | S16 | S18 | S22 | S25 | S26 | S32 | S33 | S38 | S42 | S44 | S47 | S50 | S52 | S54 | S58 | Mean |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Anti-GPIIb–IIIA** |
| Agreement (%) | 50  | —   | 88  | 25  | —   | 25  | 38  | —   | —   | 63  | 50  | —   | 25  | —   | —   | 25  | —   | 75  | 25  | 38  | 43·9 |
| Variation (%)  | 104·1| —   | 122·9| 51·2| —   | 14·9| 59·8| —   | —   | 108·1| 78·4| —   | 108·8| —   | —   | 19·7| —   | 148·1| 0·0| 25·0| 70·1|
| **Anti-GPIb–IX** |
| Variation (%)  | 33·5| —   | 79·9| 82·8| —   | 44·1| 15·2| —   | —   | 25·9| —   | —   | —   | —   | —   | —   | —   | 26·2| —   | —   | —   | 43·9 |
Control samples
For each laboratory, mean, standard deviation (SD) and the variation coefficient of the normal samples were calculated and results above the mean + 3 SD were considered positive. Given this limit, 96·0% and 97·2% of all control results for platelet-associated and plasma antibodies, respectively, were negative. Therefore of 176 control samples, only seven results for platelet-associated and five results for plasma antibodies were false positives obtained in five and four laboratories, respectively. No control sample was found positive for anti-GPIIb–IIIa or anti-GPIb–IX by more than one laboratory. The mean variation coefficient (range) of the samples of platelet-associated and plasma autoantibodies was 89·5% (11·1–272·9%) and 46·5% (21·0–78·0%), respectively.

Platelet-associated antibodies in patient samples
Platelet-associated antibodies to GPIIb–IIIa and GPIb–IX were noted by at least two laboratories in 18 and 17 of the 22 patient samples, respectively (Table II). Mean agreement for detecting platelet-associated autoantibodies against GPIIb–IIIa and GPIb–IX in the individual sample was 66·9% and 55·3%, respectively (Table III). However, overall agreement of positive results for either antibody was 74%. The mean variation coefficient (range) for anti-GPIIb–IIIa and anti-GPIb–IX antibodies was 104·8% (54·3–144·0%) and 91·0% (45·8–144·1), respectively (Table III). For anti-GPIIb–IIIa results, we found a significant overall concordance between all participating laboratories (Kendall coefficient of concordance 0·60, \( P < 0·001 \)) as well as a significant correlation between all pairs of laboratories (Spearman rank order test, range of correlation coefficient 52·3–94·0%, \( P < 0·05 \), total number of possible combination pairs 28). For anti-GPIb–IX antibodies, the overall concordance was also significant (Kendall coefficient of concordance 0·38, \( P < 0·001 \)). However, a significant correlation was noted only in 18/28 pairs of laboratories (Spearman rank order test, range of correlation coefficient 42·2–85·0%, \( P < 0·05 \)).

Plasma antibodies in patient samples
Plasma antibodies to GPIIb–IIIa and GPIb–IX were less common than platelet-associated antibodies. Positive results were found by at least two laboratories in only 12 and seven of the 22 patient samples, respectively (Table V). The mean agreement for measuring plasma antibodies against GPIIb–IIIa and GPIb–IX in the individual sample was only 43·9% and 35·9%, respectively (Table IV) and the overall agreement of positive results for either antibody was 50·1%. Furthermore, we found a significant correlation for anti-GPIIb–IIIa antibodies only between six pairs of laboratories (Spearman rank order test, range of correlation coefficient 44·4–84·0%, \( P < 0·05 \)) and none for anti-GPIb–IX antibodies.

DISCUSSION
Autoantibodies against platelet GPIIb–IIIa and GPIb–IX have been shown in a large percentage of patients with AITP.
Since AITP is diagnosed on clinical criteria, it has been suggested that detection of these autoantibodies may not be important for either diagnosis of AITP or for the management of these patients (George et al. 1994). However, measurement of these autoantibodies may prove valuable in certain clinical situations: (1) patients with additional reasons for destructive thrombocytopenia (e.g. enlarged spleen, concomitant medication), (2) thrombocytopenic patients with lymphoproliferative disorders or other neoplasms, (3) thrombocytopenic children to distinguish between acute immune thrombocytopenia and AITP, and (4) monitoring the response of AITP patients during immunosuppressive treatment (Berchtold & Wenger, 1993; Gernsheimer et al. 1989; Berchtold et al. 1989a, b).

The main advantage of the assays for anti-glycoprotein autoantibodies used in this study is the antigen-specificity attained by the use of monoclonal antibodies. However, the level of agreement among these assays has not been evaluated previously. This study was designed to evaluate the degree of agreement of the two most frequently applied methods.

The most important finding of this study was the excellent agreement of the normal control results. Using a negative-positive cut-off point of 3 standard deviations above the mean level, elevated values were found for platelet-associated and plasma antibodies in 4% and 2.8%, respectively. Therefore, as a basic condition for normal range using this limit.

Since results were reported as raw data, expressed as OD or cpm, the patient results were expressed as a binding ratio calculated by dividing the patient raw value (OD, cpm) by the mean of the 22 normal values for each laboratory. This enabled an interlaboratory comparison of the results. Unfortunately, there is no reference to determine ‘positivity’, i.e. presence of autoantibody in a single patient sample. Since no normal sample was found falsely positive by more than one laboratory, we considered a patient sample positive if elevated binding ratios were noted by at least two laboratories. In these samples there was good inter-laboratory agreement for platelet-associated antibodies. Agreement was best for anti-GP IIb–IIIa but less so for antibodies to GP Ib–IX. Why did we not achieve better agreement? Several reasons may account for this: (1) most importantly, all except two laboratories used monoclonal antibodies with different binding epitopes on the glycoproteins to immobilize the target antigens. Some monoclonal antibodies may either partially interfere with the binding of the autoantibodies or alter the protein structure (Tsukakio et al. 1987; Santoso et al. 1986). (2) Results have been reported as cpm or OD values. (3) Two different methods for antibody measurement were applied in this workshop. However, except for participant W1, agreement of results did not differ significantly among the two methods (overall agreement for MAIPA 77.5%, for IBA and MACA 83.2%).

Plasma autoantibodies to GP IIb–IIIa and GP Ib–IX were found only in a small number of samples. Moreover, interlaboratory agreement was poor and a significant correlation was noted between only six pairs of laboratories. These findings support the view that measurement of plasma autoantibodies against platelet glycoproteins is inferior to methods for platelet-associated autoantibodies in AITP. In addition, it has been shown that the specificity of plasma antibodies may differ from that of antibodies bound to platelets and that some of them may bind to cytoplasmic epitopes (Fujisawa et al. 1991, 1992). This suggests that measurement of plasma autoantibodies may be misleading in the evaluation of AITP.

In summary, we conclude that both methods used in this study give reliable and similar results for platelet-associated autoantibodies against GP IIb–IIIa and GP Ib–IX and enable an excellent distinction between normal and patient samples. In contrast, poor agreement was found when plasma autoantibodies to the same glycoproteins were evaluated. Further studies using one method as well as standardized monoclonal antibodies to immobilize target antigens are being planned.

ACKNOWLEDGMENTS

We are grateful to Dr W. Berchtold for the help with the statistical work. We also thank Evelyn Schlappritt for outstanding technical assistance.

This research is supported in part by the Swiss National Science Foundation.

REFERENCES


