The SASPA (Simultaneous Analysis of Specific Platelet Antibodies) Assay: Implementation and Performance in the Routine Laboratory Use

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Key Words

Platelet-specific antibodies · Flow cytometry

Summary

Glycoprotein-specific platelet antibodies can cause allo- or autoimmune thrombocytopenia. The specific detection of relevant antibodies is a prerequisite for diagnosis and treatment. Recently, we introduced a novel method based on the simultaneous detection and differentiation of several specific platelet IgG and IgM antibodies (SASPA) by flow cytometric analysis. It offers certain advantages compared to the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) method. The SASPA method enables a simultaneous analysis of different platelet-specific antibodies without cross-reaction and with a sensitivity comparable to MAIPA. In addition, SASPA proved to be a rapid and reliable assay and required fewer platelets in comparison to other methods. In this article we summarize the principle and benefits of the SASPA assay and report on the implementation of the SASPA method in the routine laboratory use. We also describe our 6-month experience with this assay.

Schlüsselwörter

Plättchenspezifische Antikörper · Durchflusszytometrie

Zusammenfassung

Glykoproteinspezifische Thrombozytenantikörper können eine Alloimmun- oder eine Autoimmunthrombozytopenie verursachen. Die spezifische Ermittlung der relevanten Antikörper ist unerlässlich für Diagnose und Therapie. Vor kurzem wurde von uns eine neue Methode vorgestellt, die auf der simultanen Bestimmung und Differenzierung von verschiedenen spezifischen Thrombozyten-IgM- und -IgG-Antikörpern mittels Durchflusszytometrie beruht (SASPA = simultaneous analysis of specific platelet antibodies). Verglichen mit der MAIPA(monoclonal antibody-specific immobilization of platelet antigen)-Methode bietet SASPA verschiedene Vorteile. Zum einen erlaubt SASPA die gleichzeitige Analyse von verschiedenen thrombozytenspezifischen Antikörpern ohne Kreuzreaktion und mit einer der MAIPA-Methode vergleichbaren Sensitivität. Zum anderen erwies sich SASPA als schneller und verlässlicher Assay, für den zudem im Vergleich zu anderen Methoden weniger Thrombozyten benötigt werden. In dem vorliegenden Artikel werden das Prinzip und die Vorteile des SASPA-Assays zusammenfassend dargestellt sowie die Implementierung des Assays in die tägliche Laborroutine und unsere 6-monatigen Erfahrungen mit diesem Assay erläutert.

Introduction

The investigation and detection of antibodies against platelet membrane glycoproteins (GPs) contribute to diagnosis of sev-

eral thrombocytopenic disorders, such as febrile transfusion reaction, refractoriness to platelet transfusion, autoimmune thrombocytopenia, alloimmune thrombocytopenia, post-transfusion purpura and drug-induced thrombocytopenia, and their

Fig. 1. **A** principle of the SASPA. Several human platelet-specific antibodies (hAb) bind on 3 GP complexes (GP1–GP3) of platelets (Plt). The GP-hAb complexes are incubated with 3 different mouse monoclonal antibodies (m anti-GP1–GP3) with the corresponding isotypes IgG1, IgG2a, IgG2b and form 3 trimolecular complexes. **B** After cell lysis, the lysates were incubated with beads containing 3 bead populations (B1–3) with distinct fluorescence intensities. The beads are coated with rat (R) antibodies specific against 3 distinct mouse (M) heavy-chain isotypes IgG1, IgG2a and IgG2b. These M mAbs are specific against 3 different human GPs, for example GPIa/IIa, CD32 and HLA class I, and immobilize the 3 trimolecular complexes. Human IgG (H IgG) as well as IgM (H IgM) are detected by adding goat PE- and FITC-conjugated anti-human IgG and IgM (GAHIgG, GAHIgM), respectively. The bead populations with the 3 trimolecular complexes can be easily resolved on the flow cytometer in the FL4. Accordingly, the simultaneous detection of three distinct specific human IgG as well as IgM antibodies is possible. This example shows 3 platelet-specific IgGs and 1 platelet-specific IgM.

specific therapies. In the past decades, several test systems for the detection of platelet-specific antibodies have been described, e.g. platelet immunofluorescence test (PIFT), ELISA, immunoblotting, solid-phase radioimmunoassay, fluorescence resonance energy transfer (FRET) assay, modified antigen-capture enzyme-linked immunosorbent assay (MACE), and monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay [1–9]. PIFT, for example, has been widely used as a screening test. If positive reactions occur, additional assays, e.g. ELISA, or MAIPA are used to specify the positive reactions.

The MAIPA assay first described by Kiefel et al. in 1987 [8] has been established as the standard method for platelet anti-

body detection as it maintains the GP complexes in their intact structure and thus enables the differentiation of antibody specificity. In the MAIPA assay the antibody specification is performed in a subsequent manner. The method offers a higher sensitivity and specificity than other techniques in which the patients' antibodies are tested against previously isolated platelet GPs [10]. Notably, this assay requires a large amount of platelets and the investigations on platelet antibodies have been restricted to IgG antibodies against GP Ib/IX, Ia/IIa, IIb/IIIa [11, 12]. Furthermore, in only 30–50% of patients with autoimmune thrombocytopenia and positive PIFT, specific antibodies could be detected by the MAIPA technique [13, 14]. A modified MAIPA assay has been developed to detect specific platelet antibodies for GP Ia/IIa, Ib/IX, IIb/IIIa simultaneously in patients with restricted numbers of platelets for further investigations [14]. However, this modified MAIPA assay was not able to specify the detected antibody. Moreover, the MAIPA technique has been not used routinely for the detection of IgM which can be found in 8.9% of patients with autoimmune thrombocytopenia [11]. Based on flow cytometry a fluorescence resonance energy transfer (FRET) assay was recently developed to detect specific platelet antibodies [9]. However, this test could not be introduced into routine use due to difficulties of standardization.

In 2004 we introduced a flow cytometric assay for simultaneous detection and differentiation of platelet-specific IgG and IgM antibodies (SASPA) [15]. In this article, we summarize the benefits of the SASPA method compared to the MAIPA assay and describe the implementation of the SASPA assay in routine laboratory use.

Principles and Benefits of the SASPA Method

The main difference between SASPA and MAIPA is the simultaneous use of different monoclonal antibodies (mAbs) with distinct isotypes (fig. 1) and the use of beads (Cytometric Bead Array (CBA); beads; BD Biosciences, Heidelberg, Germany) for the immobilization of mAbs. For this purpose, bead populations with distinct fluorescence intensities are mixed together. All beads are of the same size and can be easily gated in forward (FS) and side scatter (SS) by flow cytometric analysis (fig. 2). The beads are gated back in the next histogram built by the fluorescence channel 4 (FL4) channel vs. cell count. Seven bead populations are resolved in distinct peaks, and each population corresponds to a mouse heavy-chain specificity. The last 3 peaks depict 3 bead populations coated with rat monoclonal antibodies against mouse isotypes IgG1, IgG2a and IgG2b. These bead populations are considered for the analysis. Each bead population is analyzed for IgG as well as IgM antibody in the next six histograms which are built by the FL2 (phycoerythrin; PE) vs. counts or the FL1 (fluorescein isothiocyanate; FITC) for analysis of specific human IgG and IgM, respectively (fig. 2). The bead populations are coated

Fig. 2. The figure depicts a flow cytometric protocol for an analysis of a serum containing platelet-specific IgG anti-HPA-5b and -HLA class I as well as IgM anti-HLA-class I. All beads were detected in histogram 1 built by SS and FS. All beads were gated back in histogram 2 composed by the FL4 and count. In this histogram, seven bead populations were resolved in single peak. Only three bead populations immobilizing mAbs specific for human GPIa/IIa (IgG1, red), CD32 (IgG2a, blue) and HLA class I (IgG2b, green) were considered for the analysis and gated back in histograms 3, 4 and 5 or 6, 7 and 8 for analysis of IgG or IgM, respectively. Histograms 3, 4 and 5 or 6, 7 and 8 were built by FL1 (FITC) or FL2 (PE) against count, respectively, and depicted as single peaks. Each bead population is able to detect one specific human IgG as well as IgM antibody. For the evaluation, each peak was analyzed by two gates given as percentage (short gate) and MFI (long gate). The cut-off was defined for each mixture of mAb by sera of blood group AB donors.

with rat antibodies for the specific binding of the mouse isotypes IgG1, IgG2a and IgG2b. So far, we did not observe any inhibitory interference caused by the simultaneous use of three mAbs [15]. The evaluations of IgG and IgM antibodies are given as percentages and mean fluorescence intensities (MFIs). For each mixture of mAbs, the cut-off has to be defined by using serum of blood group AB donors.

As a benefit of the simultaneous detection of antibodies by SASPA, a larger number of different antibodies can be investigated in patients with low platelet counts. In the SASPA assay only 15×10^6 platelets are needed for the complete investigation on three specific antibodies, including IgG and IgM subtypes (six parameters). Moreover, the multiple antibody detection by SASPA reduced working time to perform the assay. In a comparative investigation of SASPA and MAIPA, both direct and indirect, we demonstrated the same specificity for both methods [15]. Half of the patients with platelet-associated IgG (PAIgG) determined by PIFT remained negative in the GP-specific assays focusing on GP Ia/IIa, Ib/IX, IIb/IIIa as performed to date by MAIPA [14, 16]. In addition, sensitivity of the SASPA assay was at least similar or even higher compared to the MAIPA assay. Studies on the stability of SASPA results showed lower coefficients of variation compared to MAIPA results, indicating that SASPA is more robust [15].

Implementation of the SASPA Assay in Routine Laboratory Use

The both direct and indirect SASPA assays have been established in our routine laboratory use – as direct assay it served

for the analysis of platelet-associated immunoglobulins (PAIg) and as indirect assay it is used for the determination of platelet-binding antibodies. Samples of thrombocytopenic patients are investigated as follows: i) screening by direct and indirect PIFT; ii) positive reactions in the direct PIFT are further specified by direct SASPA; iii) positive reactions in the indirect PIFT are further specified by indirect SASPA. Different combinations of three mAbs with distinct isotypes are used for routine diagnosis to simultaneously detect specific platelet antibodies (table 1). The sources for mAbs are the following: P2 specific against GPIIb/IIIa, Gi9 specific against GP Ia/IIa, SZ1 specific against GP Ib/IX, 2E1 specific against FcγRII (CD32), CLBThromb/6 specific against CD62P (all from Coulter Immunotech, Krefeld, Germany); TEA 2/16 specific against CD109 (BD Biosciences); C21 specific against human β-2 microglobulin, MM2/57 specific against CD9 (both from Cymbus Technology, Hofheim, Germany); 1A7 specific against the GPIV (Dianova, Hamburg, Germany); CLB-SW16 specific against GPV (Sanquin, Amsterdam, The Netherlands). For the investigation of IgG and IgM subclasses, we used goat anti-human IgG (PE-conjugated) and IgM (FITC-conjugated) antibodies specific against F(ab')₂ and Fc₅μ fragment, respectively (Dianova). Platelets from blood group O donors with four different homozygous HPA phenotypes (HPA-1a, -2a, -3a, -5a; HPA-1b, -2a, -3a, -5a; HPA-1a, -2a, -3b, -5a; HPA-1a, -2a, -3a, -5b) are used for the indirect PIFT and the indirect SASPA to detect platelet-binding antibodies.

In the direct SASPA assay (detection of PAIg) platelets from the patients are incubated with mixtures of three different mAbs (table 1). Dependent on the number of platelets available in patients with thrombocytopenia, we used the mAb mixtures in the sequence 1, 2, 4 and 3. After lysis of the

Fig. 3. Simultaneous analysis of specific platelet-associated IgG against GPV and GPIb/IX in a patient with autoimmune thrombocytopenia.

platelets and incubation with the CBA beads in a microtiter plate the bead-lysate complexes are washed and then incubated with goat anti-human IgG (PE-conjugated) and goat anti-human IgM (FITC-conjugated). In the final step the bead-lysate complexes are washed twice and re-suspended in PBS/BSA 1% in plastic tubes for flow cytometric analysis. As an example, figure 3 shows the detection of PAIgG against GPV and GPIb/IX in a patient with immune thrombocytopenic purpura (ITP). In fact, GPV-specific PAIgG have been suggested as common autoantibodies [14, 17–19]. For the better understanding of specific IgM in the pathogenesis of autoimmune thrombocytopenia, IgM antibodies are also analyzed in our routine setting of direct SASPA. Results concerning the detection of IgM were all negative in the case shown in figure 3.

For the detection of PAIg, positive reactions of the direct PIFT as a screening assay have only a limited value in the diagnosis of autoimmune thrombocytopenia [16, 20]. The most frequently studied target antigens of PAIgG are GP Ia/IIa, Ib/IX and IIb/IIIa [14, 16, 21, 22]. In addition, specific antibodies against CD9, CD62P, GPIV, GPV have been observed in cases of autoimmune thrombocytopenia [17–19, 23–25]. To achieve a better understanding of the pathogenesis of thrombocytopenia the investigation of further PAIg specificities is reasonable. Therefore, we included several mAbs against human CD9, CD62P, GPIV and GPV to the mixtures of the direct SASPA.

For indirect SASPA the mixtures 1, 2 and 4 (table 1) are used, dependent on the positive reactions of the patient's serum and HPA-typed donor platelets in the indirect PIFT. For example, mixtures 2 and 4 are chosen in case of positive reactions between patient's serum and platelets of a donor with the HPA-phenotype HPA-1a, -2a, -3a, -5b. In order to detect HLA class I antibody as the most common antibody together with other specific antibodies, the mAb against human β -microglobulin with the isotype IgG2b is included in mixtures 1 and 2. In mixtures 2 and 3, we included a mAb against Fc γ receptor II (CD32) to detect nonspecific binding of immunoglobulins or immune complexes [9] which can cause

Table 1. Different combinations of 3 mAbs with distinct isotypes are used in routine laboratory analysis

Mixtures	mAbs with corresponding isotypes		
	IgG1	IgG2a	IgG2b
mAb mixture 1	GPIIb/IIIa	GPIb/IX	HLA class I
mAb mixture 2	GPIa/IIa	CD32	HLA class I
mAb mixture 3	CD62P	CD32	CD9
mAb mixture 4	GPV	GPIb/IX	GPIV
mAb mixture 5	CD109	GPIb/IX	CD9

Table 2. Samples with PAIgG and PAIgM detected by direct SASPA.

IgG specificities	IgM specificities	Number of positive samples
GPIb/IX	GPIb/IX	2
GPIb/IX	–	2
GPIIb/IIIa	–	4
GPIIb/IIIa, HLA class I	–	4
GPIIb/IIIa, GPV, GPIb/IX	–	1
GPIIb/IIIa, GPIb/IX	GPIIb/IIIa	1
GPIIb/IIIa, GPIb/IX	–	1
GPIIb/IIIa, GPV, HLA class I	–	1
CD32	CD32	2

positive reactions in PIFT without a platelet-specific antibody. In suspected cases of neonatal alloimmune thrombocytopenia (NAIT) a variety of platelet GPs are involved [26–31]. Therefore, these cases are investigated with all mAb mixtures (table 1), including GPIIb/IIIa, GPIb/IX, GPIa/IIa, GPIV, GPV, CD9, CD62P and CD109 as well as HLA class I and CD32. As an example, the result of indirect SASPA from a patient's serum sample containing IgG against HPA-5b and HLA class I as well as IgM against HLA class I is given in figure 2. Three specific antibodies were simultaneously detected in a single analysis.

Table 3. Samples with platelet-binding IgG and IgM detected by indirect SASPA

IgG specificities	IgM specificities	Number of positive samples
HLA class I	–	12
HLA class I	HLA class I	1
HPA-1a	–	1
HPA-1b, HLA class I	HLA class I	1
HPA-5b	–	1
HPA-5b, HLA class I	–	1
HPA-5b, HLA class I	HLA class I	1
GPIIb/IIIa	–	1
GPIIb/IIIa	GPIIb/IIIa, HLA class I	1
GPIIb/IIIa, GPIV, HLA class I	–	1
GPIV, HLA class I	–	1
GPIa/IIa	–	1

Results of Direct and Indirect SASPA in Routine Laboratory Use

During a time period of 6 months we identified a total of 46 samples positive in the direct PIFT and 30 samples positive in the indirect PIFT. The samples were collected from patients with or without thrombocytopenia and various underlying disorders. In 18 of the 46 samples with a positive direct PIFT result we could identify specific PAIgG (table 2). Five of these samples (28%) revealed specific PAIgM as well. Anti-GPIIb/IIIa and anti-GPIb/IX were the most common PAIgs present in 16 of the 18 samples. The remaining two samples had a moderate positive result in the direct PIFT, and direct SASPA revealed positive reactions for IgG and IgM against CD32. This indicates unspecific binding of the antibodies to

the Fcγ receptor II on platelets. In 2 patients with strong positive reactions in direct PIFT, anti-GPV was detected in addition to GPIb/IX and/or GPIIb/IIIa.

In 23 of 30 samples with positive indirect PIFT, we could specify platelet-specific antibodies by the use of indirect SASPA (table 3). The analysis revealed anti-HLA class I as the most common antibody specificity. In 13 cases (57%), this was the only antibody specificity identified. Additional 6 samples (26%) showed anti-HLA class I in combination with other IgG or IgM specificities. In only 4 cases (17%) the indirect SASPA revealed a negative result for HLA class I antibodies. In 5 cases antibodies specific for platelet GPs and in another 5 cases specific HPA antibodies were found (table 3). Interestingly, in 2 of these cases anti-GPIV was identified.

Conclusion

The SASPA proved to be a simple and rapid method for the detection of platelet-specific antibodies in routine laboratory analysis and contains all advantages of MAIPA. Based on the use of flow cytometry as a sensitive method and on the selected binding of beads specific to distinct mouse isotypes, several antibodies can be analyzed simultaneously with high sensitivity and accuracy. Accordingly, a smaller amount of platelets are necessary for the evaluation. Therefore, this test system allows an extended investigation of anti-platelet antibodies in blood samples from thrombocytopenic patients which are currently not suitable for analysis with the MAIPA assay.

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