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

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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.

Introduction
The investigation and detection of antibodies against platelet membrane glycoproteins (GPs) contribute to diagnosis of several thrombocytopenic disorders, such as febrile transfusion reaction, refractoriness to platelet transfusion, autoimmune thrombocytopenia, alloimmune thrombocytopenia, post-transfusion purpura and drug-induced thrombocytopenia, and their
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 antibody 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/Ia, 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/Ia, 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 isotype. The last 3 peaks depict 3 bead populations coated with rat monoclonal antibodies against mouse isotypes IgG1, IgG2a, IgG2b. These mAbs are specific against 3 different human GPs, for example GP1α/IIα, 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-conjugated anti-human IgG and IgM (GAHlgG, GAHlgM), 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.

**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 GP1α/IIα, 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 (GAHlgG, GAHlgM), 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.
with rat antibodies for the specific binding of the mouse iso-
types 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 de-
fined 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 inves-
tigated in patients with low platelet counts. In the SASPA
assay only $15 \times 10^6$ platelets are needed for the complete in-
vestigation on three specific antibodies, including IgG and
IgM subtypes (six parameters). Moreover, the multiple anti-
body 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 re-
main 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 estab-
lished 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 pa-
tients are investigated as follows: i) screening by direct and in-
direct PIFT; ii) positive reactions in the direct PIFT are fur-
ther specified by direct SASPA; iii) positive reactions in the
indirect PIFT are further specified by indirect SASPA. Differ-
cent 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 fol-
dowing: 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 β2 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 FcγRI
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 avail-
able in patients with thrombocytopenia, we used the mAb
mixtures in the sequence 1, 2, 4 and 3. After lysis of the

**Fig. 2.** The figure depicts a flow cytometric pro-
tocol 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 his-
togram, seven bead populations were resolved
in single peak. Only three bead populations im-
mobilizing mAbs specific for human GPIa/IIa
(IgG1, red), CD32 (IgG2a, blue) and HLA class
I (IgG2b, green) were considered for the anal-
ysis 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, respect-
ively, 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.

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platelets and incubation with the CBA beads in a microtiter plate the bead-lysat 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-lysat 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 GPIa/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 Fcy receptor II (CD32) to detect nonspecific binding of immunoglobulins or immune complexes [9] which can cause 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 GPIb/IIa, 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 1. Different combinations of 3 mAbs with distinct isotypes are used in routine laboratory analysis

<table>
<thead>
<tr>
<th>Mixtures</th>
<th>mAbs with corresponding isotypes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>mAb mixture 1</td>
<td>GPIb/IIa</td>
</tr>
<tr>
<td>mAb mixture 2</td>
<td>GPIa/IIa</td>
</tr>
<tr>
<td>mAb mixture 3</td>
<td>CD62P</td>
</tr>
<tr>
<td>mAb mixture 4</td>
<td>GPV</td>
</tr>
<tr>
<td>mAb mixture 5</td>
<td>CD109</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>IgG specificities</th>
<th>IgM specificities</th>
<th>Number of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIb/IX</td>
<td>GPIb/IX</td>
<td>2</td>
</tr>
<tr>
<td>GPIb/IX</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>GPIb/IIa</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>GPIb/IIa, HLA class I</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>GPIb/IIa, GPIV, GPIb/IX</td>
<td>–</td>
<td>1</td>
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<tr>
<td>GPIb/IIa, GPIb/IX</td>
<td>GPIb/IIa</td>
<td>1</td>
</tr>
<tr>
<td>GPIb/IIa, GPIb/IX</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>GPIb/IIa, GPIV, HLA class I</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>CD32</td>
<td>CD32</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 3. Samples with platelet-binding IgG and IgM detected by indirect SASPA

<table>
<thead>
<tr>
<th>IgG specificities</th>
<th>IgM specificities</th>
<th>Number of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class I</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>HLA class I</td>
<td>HLA class I</td>
<td>1</td>
</tr>
<tr>
<td>HPA-1a</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>HPA-1b, HLA class I</td>
<td>HLA class I</td>
<td>1</td>
</tr>
<tr>
<td>HPA-5b</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>HPA-5b, HLA class I</td>
<td>HLA class I</td>
<td>1</td>
</tr>
<tr>
<td>GPIIb/IIIa</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>GPIIb/IIda</td>
<td>GPIIb/IIda, HLA class I</td>
<td>1</td>
</tr>
<tr>
<td>GPIIb/IIda, GPIV, HLA class I</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>GPIV, HLA class I</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>GPla/IIda</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

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-GPIIIb/IIa and anti-GP Ib/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 GPIIb/IX and/or GPIb/IIa. 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-GP IV 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.

Acknowledgement

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