Laboratory testing for the diagnosis of immune-mediated thrombocytopenia

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Contributions: (I) Conception and design: All authors; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: M Matsuhashi; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Alloantibodies directed against human platelet antigen (HPA) have been involved in the pathogenesis of immune-mediated thrombocytopenia, such as fetal/neonatal alloimmune thrombocytopenia (FNAIT), platelet transfusion refractoriness (PTR), and post-transfusion purpura (PTP). Therefore, for the diagnosis of these conditions, the detection and identification of the causative alloantibody is crucial. For many years, the methodologies for the detection of such antibodies were based on the use of intact platelets, however, the appropriate detection sometimes has been hampered due to the unique characteristics of the platelet membrane. In the recent years, an important progress of the serological methodologies has been observed. In this paper, we review the classical and the novel methodologies available for the detection and identification of HPA alloantibodies.

Keywords: Human platelet antigen (HPA); HPA antibody; neonatal alloimmune thrombocytopenia (NAIT); platelet transfusion refractoriness (PTR); beads-based technology; transfected cell lines; induced pluripotent stem cell-derived hematopoietic progenitor cells (iPSC-derived HPCs); endothelial cells

Introduction

Platelets play an important role in hemostasis and inflammation, and recently confirmed to be involved in innate and adaptive immune response (1,2). In addition to ABO blood group type antigens and human leukocyte antigen (HLA) class I, which are non-platelet specific antigens, human platelets also express human platelet antigens (HPA) on their surface. To date, 29 HPA systems carried on six platelet membrane glycoproteins (GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa and CD109) have been identified, and among them, 12 are grouped into six biallelic systems (HPA-1, -2, -3, -4, -5, and -15) (refer to Immuno Polymorphism Database, http://www.ebi.ac.uk/ipd/hpa/). HPAs are numbered in order of finding and are designated alphabetically in order of the frequency from high to low (3). All but one of the characterized HPAs represents single

nucleotide polymorphisms resulting in single amino acid substitution. The majority of HPAs is located on the GPIIb/IIIa (4). The frequency of HPA varies among the populations, thus different HPA are clinically relevant in different populations. Whereas anti-HPA-1a alloantibodies are the major cause of immune mediated thrombocytopenia in Caucasian, in Asian population, especially Japanese, HPA-4 and Nak^a (anti-CD36) antibodies are the predominant (5). Anti-HPA alloantibodies may be elicited by incompatible pregnancy, blood transfusion or, more rarely, by organ transplantation. HPA alloantibodies have been implicated in the pathogenesis of immune mediated thrombocytopenia, including fetal/neonatal alloimmune thrombocytopenia (FNAIT), platelet transfusion refractoriness (PTR), and post-transfusion purpura (PTP). The mechanism of FNAIT resembles that of hemolytic disease of the newborn (HDN),

in which the mother produces alloantibodies against an incompatible antigen, inherited from the father, expressed on the surface of fetal cells (6). Maternal alloantibodies of the IgG type can cross the placenta, binding to fetal platelets. The antibody-bound platelets are removed from the circulation by phagocytic cells, resulting in thrombocytopenia. The presentation of FNAIT varies from an incidentally found mild thrombocytopenia to life-threatening intracranial hemorrhage and consequent neurological sequelae (7).

PTR is defined as a poor response to at least two consecutive platelet transfusions. PTR patients fail to achieve the appropriate platelet count increment because of anti-platelet antibodies produced by repeated blood transfusions (8,9). The most frequent alloantibodies involved in immunological PTR are anti-HLA class I antibodies (around 80–90%), followed by anti-HPA antibodies (around 10–20%) (10,11). Although platelet transfusions are necessary to prevent hemorrhagic complications, the onset of PTR during chemotherapy or during the regimen for hematopoietic stem cell transplant, increase the risk of hemorrhage and threaten the life of patients. Therefore, the detection and identification of the causative antibodies is crucial for the diagnosis, prevention and management of immune-mediated thrombocytopenia.

In this review, we will describe the presently available methods for the detection of anti-platelet antibodies and discuss on the advantages and disadvantages of each methodology.

Classical methods

Various techniques have been developed for the serological investigation of platelet immune disorders (*Table 1*). The earliest assays, such as platelet aggregation assay, platelet factor 3 (PF-3) release test and serotonin release test, were based on the platelet function-dependent endpoints (12,13). However, their sensitivity was low, because some antibodies elicit platelet activation, but most of them do not. Thereafter, binding assays using intact platelets have been developed, including platelet immuno-fluorescence test (PIFT) and mixed-passive hemagglutination assay (MPHA), which are described below. However, it is difficult to discriminate between platelet specific antigens and non-platelet specific antigens in these assays. In the 1980's, GP specific antibodies were produced, which contributed for the development of antigen capture assays such as antigen capture ELISA (ACE), modified antigen-capture ELISA

(MACE), and monoclonal antibody-specific immobilization of platelet antigen (MAIPA). These antigen-capture assays allow the identification of the antigenic epitopes on the specific GPs.

PIFT

In the PIFT, intact platelets are incubated with the patient's or the control serum and allowed to bind to the antigenic epitopes. Then, fluorescence-labeled anti-human IgG/IgM are added as the secondary antibody, and allowed to bind to the antibody bound to the antigenic epitope. The fluorescence-labeled platelets are then analyzed by fluorescence microscopy or by flow-cytometry (*Figure 1*). Although initially the fluorescence microscope was applied for the evaluation of the fluorescence intensity of the stained platelets, recently, it has been replaced by flow-cytometry because of the lack of the objectivity of the former (14). The measurement of the fluorescence intensity by flow-cytometer allows the more objective interpretation of the data. The result can be quantitatively expressed and judged by calculating the ratio of the fluorescence intensity of samples to that of the negative control. The flow-cytometry method is one of the widely applied techniques based on the use of intact platelets, and it is highly sensitive for the detection of most of the anti-HPA antibodies, except for HPA-5 and HPA-15 antibodies, which are expressed at lower levels on the platelet surface. Around 3,000–5,000 antigenic sites of HPA-5, and only 1,000 sites of HPA-15 are expressed on platelets (4). The detection of such low expression antigens by this method is challenging. The binding assays are associated with the issue of difficult identification of multiple antibodies, especially the coexistence of anti-HLA with anti-HPA antibodies. For the elimination of the reactivity of anti-HLA antibodies, treatment of platelets with chloroquine or acid can be used, which cause destruction of the β_2 microglobulin on the surface of platelets. However, the complete elimination of the reactivity with anti-HLA antibodies may be difficult in case of a coexisting high titer anti-HLA antibody in the serum (15,16). Also, anti-A and anti-B antibodies are detected, so blood group O platelets are chosen for the assay.

MPHA and magnetic-MPHA

In MPHA, intact platelets or platelet membrane extracts are coated to the wells of the round-bottom microtiter

Table 1 Advances of platelet antibody assays

Generation	Principle	Assays
First	Platelet function	Platelet aggregation
		Complement fixation
		Platelet factor 3 (PF-3) release test
		Serotonin release test
Second	Measurement of PAIgG and PBIgG	Radioimmunoassay
		Immunofluorescence test
		Mixed passive hemagglutination (MPHA)
		Solid-phase RBC adherence (SPRCA)
		Enzyme-linked immunosorbent assay (ELISA)
Third	Use of platelet glycoproteins as targets	Immunobead assay
		Immunoblotting
		Monoclonal antibody immobilization of platelet antigens (MAIPA)
		Antigen capture ELISA (ACE)
		Modified antigen capture ELISA (MACE)
Fourth	–	Beads based method
		❖ Simultaneous analysis of specific platelet antibodies (SASPA)
		❖ Immune-complex capture fluorescence analysis (ICFA)
		❖ Platelet antibody beads array (PABA)
		❖ PAKLX (commercial kit)
		Application of new tools
		❖ Transfected cell lines
		❖ iPSC-derived HPCs expressing allele-specific forms of HPA
		❖ Endothelial cells

iPSC, induced pluripotent stem cell; HPC, hematopoietic progenitor cell; PAIgG, platelet-associated IgG; PbIgG, platelet-binding IgG; RBC, red blood cell.
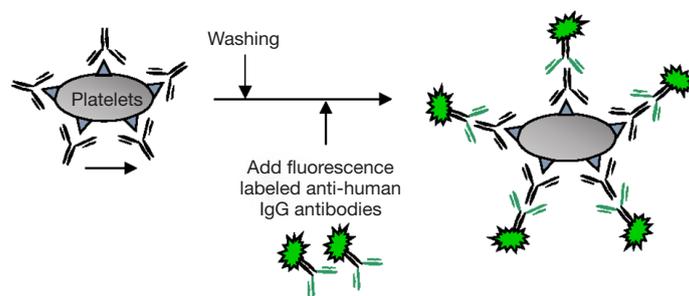
Figure 1 The principle of PIFT. Platelets are incubated with the anti-platelet antibody and allowed to bind the antigenic epitope, then incubated with the secondary fluorescence-labeled anti-human IgG. The fluorescence intensity on the surface of platelets is measured by a flow-cytometer. PIFT, platelet immuno-fluorescence test.
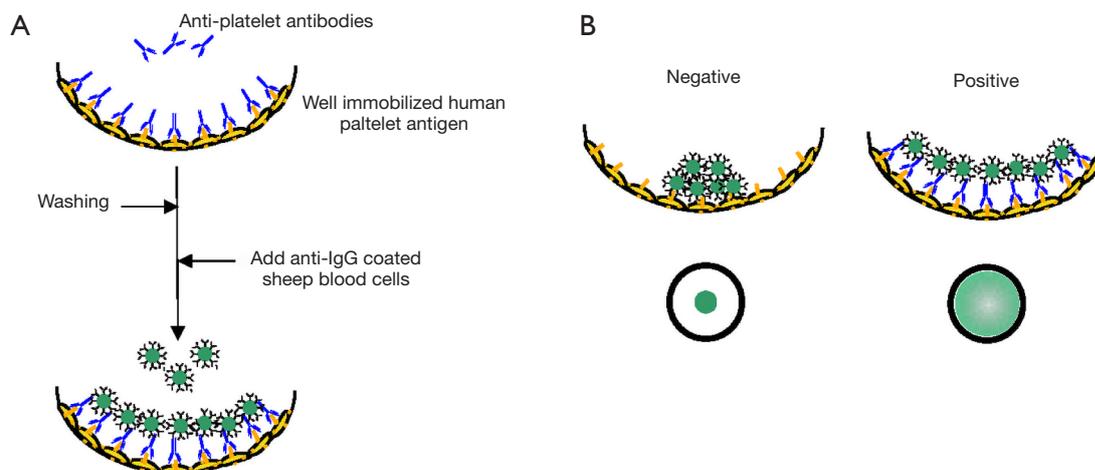
Figure 2 The principle of the MPHA assay and the judgement of results. (A) Intact (or extracts of) platelets are coated to the bottom of the round wells of a microtiter plate, and incubated with anti-platelet antibodies. Then, indicator cells, consisting of either sheep red cells (MPHA) or magnetic beads (M-MPHA) coated with anti-human IgG, are seeded and allowed to sediment spontaneously (MPHA), or are pulled down by a magnetic plate (M-MPHA). (B) In the positive reaction (right), the indicator cells are bound to the platelets and do not sediment, whereas in the negative reaction (left), they sediment to the bottom of the wells, forming a clear ring. MPHA, mixed-passive hemagglutination assay.

plates, and human antisera is added to the microtiter wells to allow binding to the platelet antigens (17-19). For the detection of the reaction, indicator cells, consisting of sheep red cells (MPHA) or magnetic beads (M-MPHA), coated with anti-human IgG, are added to the wells and allowed to sediment spontaneously (MPHA) or pulled-down by a magnetic plate (M-MPHA). As shown in *Figure 2*, in case anti-platelet antibodies are present, they bind to the correspondent antigens immobilized to the bottom of the microtiter wells, and are recognized by the anti-human IgG present on the surface of indicator cells. In case of a positive reaction, the indicator cells bind to the human IgG bound to the specific antigens and consequently remain disperse, thus, do not sediment. In the negative reaction, the indicator cells sediment to the bottom of the wells, creating a clear "ring". For the judgement of the reaction, sample wells are compared with the pattern of the negative control wells. The MPHA has high sensitivity for most HPA antibodies, including HPA-5, and is largely used in Japan for the platelet serological testing. It has many advantages, including the handling of various samples in a single assay, easy manipulability, and the very short time of the assay, especially with magnetic beads, which allows the reaction to develop in about 3 min after the antibody binding to platelet antigens. The main limitation of this method is the interference of the antibody identification

by a coexistent strong HLA antibody. The treatment with chloroquine or acid, as described in PIFT, can remove HLA class I antigenicity, however, some antigenicities, such as HPA-15 antigen, are also destroyed by this treatment.

Antigen capture assays

Presently, there are three types of antigen capture assay; ACE, modified ACE (MACE) and MAIPA (20-22). These methods differ in the way the glycoprotein antigens are captured, as shown in *Figure 3*. MAIPA is widely used in Europe and other countries, whereas MACE is preferred in the U.S. (23,24).

In the MAIPA, platelets are sensitized with patient's serum, washed, and then incubated with a mouse monoclonal antibody recognizing the desired target glycoprotein on the platelet surface. After sensitization with both antibodies, platelets are washed and solubilized using a nonionic detergent such as Triton. After centrifugation to remove cytoskeletal fragments, an aliquot of the supernatant lysate containing the trimolecular complex, which consists of GPs-specific mAb/GPs/anti-platelet antibody, is added to the wells of the microtiter plate previously immobilized with goat anti-mouse IgG. This immobilized IgG captures the complex. For the detection of the captured complex, a peroxidase-labeled anti-human IgG is added, followed
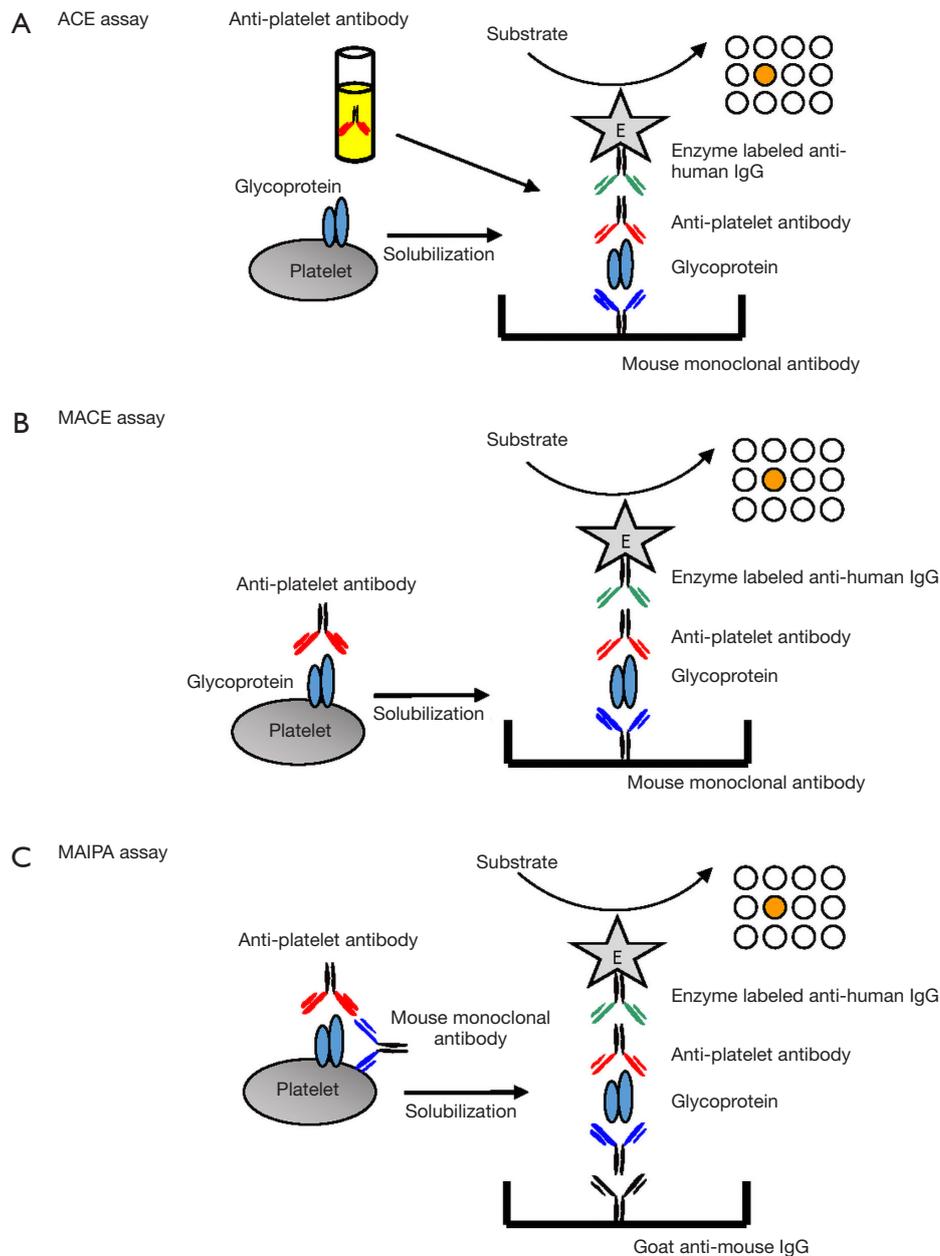
Figure 3 The principle of antigen capture assays. (A) ACE assay: the platelet lysate containing membrane glycoproteins (GPs) is added to the wells of a microtiter plate coated with GPs-specific antibodies, which captures the specific GPs. The well is then washed and incubated with the anti-platelet antibody. The anti-platelet antibody bound to the glycoprotein is detected by the addition of a peroxidase-labeled anti-human IgG, followed by an appropriate substrate. (B) MACE assay: platelets are incubated with anti-platelet antibodies, and then lysed. The complex consisting of GPs/anti-platelet antibody is added to the well of a microtiter plate coated with GPs-specific mAb, which captures the complex, and the captured complex is detected by the addition of a peroxidase-labeled anti-human IgG, followed by an appropriate substrate. (C) MAIPA assay: platelets are reacted with both anti-platelet antibodies and the GP-specific mAb, and then solubilized. The complex consisting of GPs-specific mAb/GPs/anti-platelet antibody is added to the well of a microtiter plate coated with anti-mouse IgG, which captures the complex, and the captured complex is detected by the addition of a peroxidase-labeled anti-human IgG, followed by an appropriate substrate.
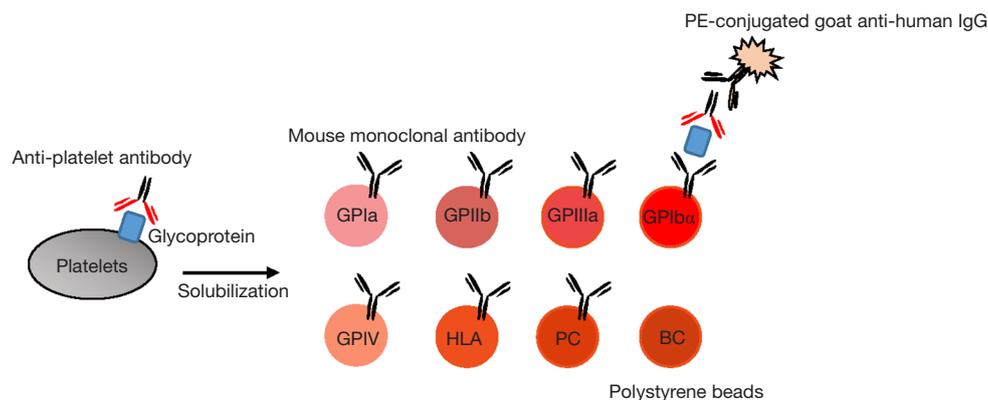
Figure 4 The principle of immune-complex capture fluorescence analysis (ICFA). Platelets are incubated with the anti-platelet antibodies and then lysed. The complex consisting of GPs/anti-platelet antibody is incubated with the polystyrene beads immobilized with GPs-specific mAb, which captures the complex, and then incubated with PE-conjugated goat anti-human IgG antibodies. The beads are read on the Luminex system (Luminex Co, Austin, TX, USA). PC, positive control beads; BC, background control beads.

by an appropriate substrate. This method is highly sensitive and specific, and considered the gold standard method in platelet immunology. The antigen capture methods, including MAIPA, allow the discrimination of HPA and HLA antibodies. However, the selection of the mouse monoclonal antibodies is important, because some monoclonal antibodies may compete with anti-HPA alloantibodies, especially Nak[a] antibodies, in binding to the antigenic epitope (25). It is not appropriate as a screening test, because of the time-consuming protocol, and the need of multiple microplates and large panels of HPA-typed platelets in order to identify antibodies reactive with HPA located on all the major GPs. The MAIPA protocol and reagents have been modified in different laboratories; therefore, there are inter-laboratory variations of the sensitivity for the detection of HPA antibodies (26,27).

New approaches for the detection of HPA antibodies

Beads-based technologies

Recently, several different beads-based techniques, which are highly sensitivity and have high throughput, have been developed. As anti-HPA-1a antibodies are the most clinically relevant among Caucasian, beads-based assays focused on the detection of HPA-1a antibodies have been reported (28-30). However, presently, it is also available for the testing of the major HPA antibodies (31-34). The great advantage of the beads-based technologies is the feasibility

of simultaneous analysis of various antibody specificities in a single tube or well. Additionally, these assays eliminate the laborious and time-consuming work, which are the major disadvantages of the classical methods. These technologies are known to be associated with the detection of antibodies with low clinical relevance or naturally occurring antibodies when applied for HLA antibody testing (35,36), dependent on the use of recombinant HLA antigens. However, presently, there is no evidence of such issues when these technologies are applied for the detection of anti-HPA antibodies.

Immune-complex capture fluorescence analysis (ICFA)

ICFA is a methodology based on the Luminex technology combined with the antigen capture method (32). Platelets are sensitized with patient's serum, then washed and solubilized with a nonionic detergent, similar to the MACE assay. Then, the aliquot of the supernatant lysate, obtained after centrifugation, is incubated with the polystyrene beads coupled with GP specific mouse monoclonal antibodies, instead of microtiter plate in MACE. The beads are then washed and incubated with PE-conjugated goat anti-human IgG antibodies. After washing, the beads are read on the Luminex system (Luminex Co, Austin, TX, USA) (*Figure 4*). This method requires a repertoire of donor platelets for the detection of HPA and HLA antibodies, however at a lower volume than in classical methods. In the previous report, the detection of HPA-1a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6b and Nak[a] antibodies by this technology has been confirmed, but HPA-15 antibodies were not tested because of the lack

of anti-sera (32). Similar methodologies, such as the platelet antibody beads array (PABA), have been reported (37), and applied for the detection of HPA-15b alloantibodies. Cell lines expressing recombinant CD109 proteins, and fresh and frozen HPA-15 typed platelets were tested, but only the cell lines and the fresh platelets were able to identify HPA-15b antibodies. As the ICFA assay is based on antigen capture assay, the selection of the capture monoclonal antibodies is essential for the prevention of false negative reactions.

PAKLx

PAKLx (Lifecodes, HOROGIC/Genprobe) is a beads-based method for the detection and identification of anti-platelet antibodies. In this kit, purified GPs from HPA-typed platelet donors and HLA class I antigen from pooled platelets, consisting of 100 Caucasian, 100 African American, and 100 Hispanic blood donors, are immobilized to the polystyrene beads. The beads are sensitized with patient serum, followed by washing. Then, beads are incubated with PE-labeled anti-human IgG antibodies, and the mean fluorescence intensity (MFI) of the beads is measured using the Luminex equipment. The MFI of each sample bead is compared with that of negative control beads, and the results judged as negative or positive. Presently, HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b and Nak^a antibodies can be detected, but not anti-HPA-15a and -15b antibodies, because HPA-15 antibody identification beads are not available in the kit (33). It is well known that anti-HPA-15 antibodies are clinically significant in NAIT and PTR, thus, the beads assay including anti-HPA-15 antibody detection is greatly desired. Because of the easy manipulability, not requiring especial skills, it is a good screening method. Additionally, HPA-typed platelets are not required and only very low amount of sera is enough for the testing. It is also reported to have a high sensitivity. However, it has the disadvantage of not been able to detect some antibody specificities, such as anti-HPA-3a, which could be detected only by the PIFT and MAIPA using appropriate monoclonal antibodies, and -5b antibodies (33). In addition, it could not detect low titer and low avidity antibodies. Therefore, they should be used in combination with other methods.

Transfected cell lines, induced pluripotent stem cell (iPSC)-derived hematopoietic progenitor cells (HPCs) expressing allele-specific forms of HPA and endothelial cells (ECs)

An important issue in the laboratory diagnosis of platelet alloantibodies is the difficulty in obtaining the appropriate platelets, especially those expressing the low frequency HPA antigens.

In the last decade, the stably transfected Chinese hamster ovary (CHO) cell lines expressing HPAs were generated. The validity of these cell lines was confirmed by MAIPA; however, it was observed that the expression level of HPAs on the cells was reduced over a period of time (38,39). Recently, the HPA panels of transfected K562 cells, which do not express HLA or HPA, have been developed. The reactivity of these cells with HPA antibodies is very specific and sensitive, and no reactivity with normal human sera was confirmed. Until now, the transfected cell lines of HPA-1a, -1b, -2a, -2b, -3b, -4b, -5b, -6b, -7b, -7variant, -12a, -13b, -15a, -15b, -18b, -21b, and CD36 have been developed (40). Transfectant cells are valuable tools for the detection and identification of HPA antibodies, since they allow the identification of single specificities even in samples containing concomitant HLA antibodies, which hamper the antibody identification in the binding assays such as PIFT and MPHA.

More recently, the group from Milwaukee reported on the successful production of pluripotent stem cells (iPSCs) from the megakaryocyte-like DAMI cells, using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) gene-editing technology (41). They were able to produce cells expressing the HPA-1b alloantigenic epitope, which is the low frequency antigen of HPA-1. These designer platelets are promising tools for the diagnostic, investigative and ultimately therapeutic use in clinical conditions associated with platelet alloantibodies.

In addition, an important target of anti-HPA alloantibodies seems to be the GPIIIa (integrin β3), which is also present on ECs in a complex with the integrin alpha v (αvβ3). Alloantibodies reactive with the αvβ3 may play an important role in the pathogenesis of intracranial hemorrhage (ICH), the most severe complication of FNAIT (42,43). Antibody against β3 integrin, but not GPIbα, reduced brain and retina vessel density, impaired angiogenic signaling, and increased EC apoptosis, in a model of FNAIT (42). Using serum samples from mothers of FNAIT with and without ICH, it was confirmed that anti-HPA-1a antibodies of anti-αvβ3 specificity were present among those FNAIT with ICH, and these antibodies induced EC apoptosis of HPA-1a positive ECs by caspase-3/7 activation, mediated by reactive oxygen species and interfered with EC adhesion to vitronectin and with EC tube formation (43).

From these results, it can be suggested that the identification of those alloantibodies of anti-αvβ3 specificity may contribute for the determination of severe cases of FNAIT, and may help implementing the early and more aggressive preventive measures.

All these cell lines can be applied not only in the classical methods, including MAIPA, MACE and immunofluorescence test, but also in the novel technologies such as ICFA, and their application will open new premises for the appropriate diagnosis of immune thrombocytopenia. The use of ECs as the target will be important for the diagnosis of severe cases of FNAIT, especially those associated with ICH.

Conclusions

New methodologies for the detection of anti-platelet antibodies have been developed, and the validation studies have been conducted. However, since the number of the serum samples used in the validation test is limited, as well as the antibody specificity, and some discrepancies between the classical and the novel methods have been confirmed, further investigation is required to validate these novel technologies. Most alloantibodies directed against the major HPAs shall be detected using the existing technology. However, the detection of some antibodies, such as anti-HPA-3, is still problematic. Previous reports indicated there may be different structural requirements of the HPA-3 epitopes, some antibodies being detected only when whole platelets are used, and not with the beads-based technology (33,44,45). Additionally, the groups from Germany and Milwaukee reported that surface plasmon resonance (SPR) allowed to detect the low-avidity anti-HPA-1a antibodies involved in severe FNAIT cases, which were not identified by the MAIPA assay (46,47). Recent reports indicated that low-avidity anti-HPA-1a antibodies are present in a large number of maternal sera, in which antibodies were not previously detected by the MAIPA assay (47,48). Presently, no single technique alone is sufficient to detect all clinically relevant alloantibodies, because of the complexity of platelet membrane glycoproteins carrying platelet alloantigenic determinants, and the nature of the alloantibodies themselves. Many low frequency HPAs have been found in the last years (49), and it is believed that new HPA antigens will continue to be identified in the future. By applying these novel beads-based assays, alloantibodies against new or low frequency HPA antigens, against conformation-dependent antigenic determinants formed only on intact

platelets, or against endothelial specific epitopes may be overlooked. Although significant progress has been made in platelet serology, the advantages and disadvantages of each method should be taken into account for the further improvement of the technologies for platelet antibody detection.

Acknowledgments

Footnote

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