Review

Detection and identification of platelet antibodies and antigens in the clinical laboratory

B.R. Curtis and J.G. McFarland

As a result of the unique functional properties of platelets, more-robust methods were required for detection of antibodies raised against them. Immunofluorescence detection by flow cytometry, solid-phase red cell adherence, and antigen capture ELISAs are some of the current tests that have been developed to meet the challenges of platelet antibody detection and identification and antigen phenotyping. Recently developed protein liquid bead arrays are becoming the next-generation platelet antibody tests. Fueled by development of PCR and determination of the molecular basis of the Pl^{A1} human platelet antigen (HPA), serologic platelet typing has now been replaced by genotyping of DNA. Allele-specific PCR, melting curve analysis, and 5′-nuclease assays are now evolving into more high-throughput molecular tests. Laboratory testing for the diagnosis of immune platelet disorders has advanced considerably from its humble beginnings.
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Historic Background

Development of suitable tests for detection and identification of platelet antibodies and antigens in the clinical laboratory diagnosis of immune platelet disorders proved difficult compared with the relative ease with which simple tests were designed for detection of RBC antibodies and antigens. Homotypic platelet–platelet interactions via cell surface receptors and their ligands caused nonspecific platelet agglutination after centrifugation, thereby preventing the application of standard RBC antibody tube agglutination tests in the detection of platelet antibodies. In addition, isolation and preparation of platelets for testing resulted in elevated cell surface immunoglobulin levels that significantly reduced assay sensitivity and specificity.[1]

Early assays for detection of platelet antibodies used the functional properties of platelets as assay end points such as protein receptor–induced activation, alpha and dense granule release, and platelet aggregation and agglutination. These tests are often referred to as phase I or first-generation assays and will not be discussed further. Later, more sophisticated tests (phase II or second-generation assays) included direct or indirect measurements of platelet surface-bound immunoglobulins using secondary antibody reagents in immunoblots,[2] RIA,[2–4] ELISA,[5–7] immunofluorescence,[8–10] latex agglutination,[11,12] and solid-phase RBC adherence.[13,14] With the production of platelet glycoprotein (GP)-specific monoclonal antibodies throughout the early 1980s, phase III or third-generation assays were developed that allowed for detection of antibodies bound to platelet membrane GPs captured in the wells of microtiter plates.[15–18] More recently, assays using purified platelet GPs covalently linked to individual polystyrene beads have been developed that greatly increase assay throughput. These same antibody detection assays were also used to phenotype platelets for the human platelet antigens (HPAs) expressed. However, serologic typing required the availability of relatively large quantities of highly specific typing sera free of HLA class I antibodies. With the cloning and identification of the HPA-1a (Pl^{A1}) gene,[19] serologic typing was soon replaced by molecular methods for genotyping DNA.[19,20]

Platelet Glycoproteins and Antigens

Like RBCs, platelets have their own unique surface antigens. To date, 28 different platelet-specific antigens carried on five different GPs have been completely characterized. The Platelet Antigen Nomenclature Working Party of the International Society of Blood Transfusion and International Society of Thrombosis and Hemostasis[21] has assigned 23 HPA-designated systems (Table 1). With the exception of one (HPA-14bw), all consist of two alleles with the higher frequency allele given the "a" designation and the lower frequency allele the "b" designation. A "w" (workshop) designation has been assigned to antibodies for which the antithetical allelic antigen has not yet been found. Platelets also express other "non-HPA" antigens to which antibodies are produced that have been implicated in immune platelet disorders including the blood group ABO(H) antigens, Nak(a) antigen on GPIV, and HLA class I (Table 1). A, B, and H antigens are expressed on virtually all major platelet membrane GPs, but the majority are expressed on GPIIb and platelet endothelial cell adhesion molecule (PECAM or CD31).[22] HLA class I A, B, and C antigens are all expressed on platelets, which are the major source of HLA class I antigen in the blood.[23]

Immune Platelet Disorders Evaluated by Platelet Immunology Laboratories

Platelet antibody and antigen tests are primarily used in the laboratory investigation of six immune platelet disorders: (1) autoimmune thrombocytopenia, often referred to as ITP (idiopathic thrombocytopenic purpura), in which patients produce antibodies against their own platelets; (2) neonatal alloimmune thrombocytopenia (NATP), in which maternal IgG antibodies produced against a paternal platelet antigen inherited by the child cause destruction of fetal and newborn platelets; (3) posttransfusion purpura (PTP), an acute, severe thrombocytopenia that develops 5 to 10

Table 1. Platelet Glycoproteins and Antigens

Antigens	Other Names	Phenotypic Frequency*	Glycoprotein Location/ Amino Acid Change	Nucleotide Substitution
HPA-1a (Pl[A1]) HPA-1b (Pl[A2])	Pl[A], Zw	72% a/a 26% a/b 2% b/b	GPIIIa / L33P	T>C 196
HPA-2a (Ko[b]) HPA-2b (Ko[a])	Ko, Sib	85% a/a 14% a/b 1% b/b	$GPIb_{\alpha}$ / T145M	C>T 524
HPA-3a (Bak[a]) HPA-3b (Bak[b])	Bak, Lek	37% a/a 48% a/b 15% b/b	GPIIb / I843S	T>G 2622
HPA-4a (Pen[a]) HPA-4b (Pen[b])	Pen, Yuk	>99.9% a/a <0.1% a/b <0.1% b/b	GPIIIa / R143Q	G>A 526
HPA-5a (Br[b]) HPA-5b (Br[a])	Br, Hc, Zav	80% a/a 19% a/b 1% b/b	GPIa / E505K	G>A 1648
HPA-6bw	Ca[a], Tu	<1% b/b	GPIIIa / R489Q	A>G 1564
HPA-7bw	Mo[b]	<1% b/b	GPIIIa / P407A	G>C 1317
HPA-8bw	Sr[a]	<0.1% b/b	GPIIIa / R636C	T>C 2004
HPA-9bw	Max[a]	<1% b/b	GPIIb / V837M	A>G 2603
HPA-10bw	La[a]	1% b/b	GPIIIa / R62Q	A>G 281
HPA-11bw	Gro[a]	<0.5% b/b	GPIIIa / R633H	A>G 1996
HPA-12bw	Iy[a]	1% b/b	$GPIb_{\beta}$ / G15E	A>G 141
HPA13bw	Sit[a]	<1% b/b	GPIa / M799T	T>C 2531
HPA-14bw	Oe[a]	1%	GPIIIa / De167K	AAG 1929-31
HPA-15a (Gov[b]) HPA-15b (Gov[a])	Gov	35% a/a 42% a/b 23% b/b	CD109 / Y703S	A>C 2108
HPA-16bw	Duv[a]	<1%	GPIIIa / T140I	C>T 517
HPA-17bw	Va[a]	<1%	GPIIIa / T195M	C>T 622
HPA- ?	Cab[a]	<1%	GPIa / Q716H	G>T 2235
HPA- ?	Sta	<1%	GPIIIa / K137Q	A>C 487
HPA- ?	Kno	<1%	GPIIb / T619M	C>T 1949
HPA- ?	Nos	<1%	GPIIIa / E628K	G>A 1960
NA	Nak[a]	99.8% (Caucasian) 97% (African) 96% (Asian)	CD36 (GPIV)	T>G 1264 C>T 478
NA	A, B, H	42% A 10% B 44% O (H) 4% AB	GPIIb, PECAM, GPIIIa GPIa, GPIIa, GPIV	NA
NA	A, B, C	NA	HLA class I	NA

*Phenotypic frequencies for the antigens shown are for the Caucasian population. Significant differences in gene frequencies may be found in African and Asian populations. NA = not applicable; ? = HPA number not yet assigned

days after a blood transfusion in a patient previously sensitized to a platelet alloantigen during pregnancy or prior transfusion that leads to autoantibody formation; (4) multiplatelet transfusion refractoriness (MPTR), which occurs most frequently in oncology patients receiving platelet transfusion support after bone marrow transplant or chemotherapy who become highly alloimmunized, primarily to HLA class I antigens; (5) drug-induced thrombocytopenia (DITP), in which patients develop acute, severe thrombocytopenia caused by drug-dependent antibodies (DDAbs) that develop after exposure to various drugs—some of the drugs that most commonly cause DDAbs are quinine, vancomycin, and GPIIb/IIIa inhibitors; and (6) heparin-induced thrombocytopenia with thrombosis (HITT) or without it (HIT)—thrombocytopenia develops most often in patients exposed to unfractionated heparin, resulting in the production of antibodies to platelet-factor 4 (PF4)—heparin complexes that bind to platelet Fc receptors, leading to platelet activation and clearance.

Detailed descriptions of various methods for detection of platelet antibodies and allelic gene polymorphisms that encode platelet antigens are described in this article. The role of platelet antibody testing in the diagnosis of the various immune platelet disorders is explored in other articles in this issue.

Platelet Antibody Tests Currently Used for the Laboratory Investigation of Immune Platelet Disorders

Assays using intact platelets

Despite a good selection of platelet antibody detection methods available today, no single method is sufficient.[24] Each method has limitations that make it necessary to perform a combination of several methods to ensure a thorough antibody workup. Specialized platelet antibody reference laboratories typically perform an assay that uses intact platelets to screen serum or plasma for antibodies, a GP antigen capture assay for identification of the antigen that antibodies target, and genotyping of patient DNA to help confirm the HPA specificity of platelet alloantibodies identified.

Flow cytometry

The platelet suspension immunofluorescence test (PSIFT) using a fluorescence microscope developed by von dem Borne et al. in 1978[8] was the first widely adopted method using intact platelets for the detection of platelet-reactive antibodies. A flow cytometric version of the PSIFT was also described in von dem Borne's original report, and as the instruments became more available, immunofluorescence by flow cytometry has now become one of the most popular and sensitive methods for detection of platelet antibodies using intact platelets.[9,10,25] A typical assay includes incubation of patient's serum or plasma with platelets for 30 to 60 minutes, usually at room temperature. The platelets are then washed to remove unbound and some nonspecifically bound immunoglobulins, followed by incubation with fluorescent labeled $F(ab')_2$ fragment of polyclonal anti-human IgG for about 20 minutes in the dark. After one wash, the platelets are suspended in buffer, and platelet-bound fluorescence is detected by a flow cytometer. Mean or median fluorescence intensity (MFI) values and fluorescence histograms are generated by the flow cytometer software for use in determining results (Fig. 1). Results can be expressed as a fluorescence ratio (FLR) of MFI obtained with the patient's serum divided by MFI obtained with a negative control serum from a normal healthy individual incubated with the same platelet target. An FLR cutoff for a positive interpretation can be established by statistical analysis of FLR values obtained from testing 20 to 30 normal sera. Serum or plasma positive control samples should include IgG and IgM platelet-reactive antibodies detected in previously tested patient samples.

FITC is the most commonly used fluorescent label for the anti-IgG probe. The fluorochrome phycoerythrin (PE) attached to an anti-human IgM probe is often used for simultaneous detection of IgM platelet antibodies. The two labeled probes can be added together during the final incubation, and both activated to fluoresce by the 480-nm laser. The fluorescent signals are detected separately

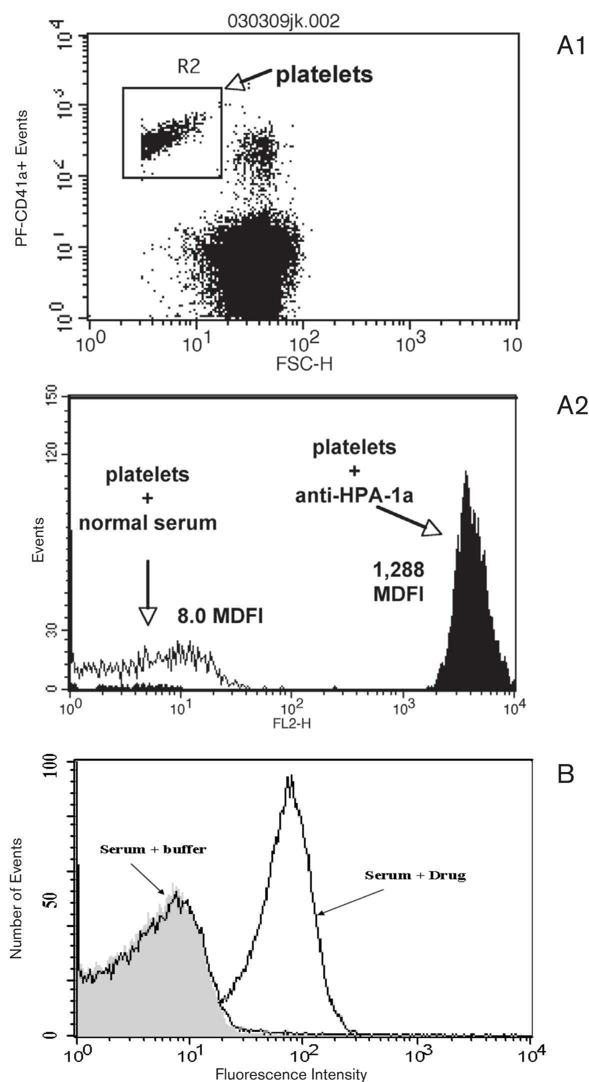

Fig. 1 Flow cytometry fluorescence dot plot (**A1**) and histograms of platelet antibody test results (**A2, and B**). (**A**) Whole blood was incubated with a phycoerythrin (PE)-labeled monoclonal antibody specific for platelet GPIIb (PE-CD41a), and normal or patient serum with HPA-1a antibodies. Platelets were then washed, and bound human IgG platelet antibodies were detected by incubation with FITC-$(Fab')_2$ fragment of goat anti-human IgG and detection of platelet-bound fluorescence by flow cytometry. Platelets were identified by gating on PE-CD41a+ fluorescent cells in dot plot (**A1**), and level of FITC-anti-human IgG fluorescence determined in histogram (**A2**) displayed for both normal serum (white) and anti-HPPA-1a serum (black). MDFI = median fluorescence intensity. (**B**) Histograms of drug-dependent platelet antibody test. Platelets were incubated with patient serum in the presence (white histogram) and absence (gray histogram) of drug, and antibody detected with FITC-$(Fab')_2$ fragment of goat anti-human IgG. Fluorescence is significantly stronger in the presence of drug, indicating the patient has drug-dependent platelet antibodies.

because their emission wavelengths differ. $F(ab')_2$ fragments (antibody with Fc end removed) of the anti-immunoglobulin probes are used to prevent nonspecific binding via Fc receptors on the target platelets.

Flow cytometry has proved to be a very sensitive method for detection of platelet antibodies and is considered the best method for detection of drug-dependent platelet antibodies induced by drugs like quinine,[26] sulfamethoxazole,[27] and vancomycin.[28] For detection of drug-dependent antibodies the previously described procedure can be used with inclusion of a second tube of platelets and serum incubated in the presence of the implicated drug at a concentration between 1 and 10 mM (Fig. 1).[29,30] It is vital that the tube of platelets with patient's serum and drug be washed with drug solution to preserve platelet-bound drug-dependent antibodies. An FLR is calculated by dividing the MFI obtained with patient's serum in the presence of drug by the MFI obtained with patient's serum tested with only buffer. FLR ratios of 2.0 or greater are usually considered positive for drug-dependent antibodies.

Flow cytometry can also be used for detection of platelet-associated IgG (PAIgG) and IgM in suspected cases of ITP.[31–33] The patient's platelets are simply isolated, washed, and incubated with FITC-anti-IgG and PE-anti-IgM; fluorescence is detected by flow cytometry.[34] The method is sensitive, simple, and relatively quick to perform, but has lower specificity owing to nonspecifically bound platelet immunoglobulins like immune complexes that can be present on platelets of patients with nonimmune thrombocytopenias.

With the adaptation of performing platelet antibody flow cytometry tests in 96-well microtiter plates, the use of frozen-stored platelets, and the availability of flow cytometers with high-throughput sampler (HTS) attachments, flow cytometry has become a quick, high-volume method for platelet antibody detection, including as a method for platelet crossmatching to select compatible platelets for transfusion to highly alloimmunized thrombocytopenic patients.[35,36] Disadvantages include the requirement for an expensive instrument, specialized reagents, and the inability to determine antibody HPA specificity in the presence of non-HPA antibodies, e.g., HLA class I.

Solid-phase RBC adherence

Another method for detection of platelet antibodies that uses intact platelets is the solid-phase RBC adherence (SPRCA) or mixed-passive hemagglutination assay (MPHA), first developed by Shibata et al.[13] and later made available as a commercial kit (Capture-P, Immucor, Inc., Norcross, GA).[14,37] SPRCA uses intact target platelets adhered to the wells of a round-bottom 96-well microtiter plate. Patient test serum or plasma is added to the wells and incubated, followed by repeated washing of the wells. Platelet-bound antibodies are detected by addition of indicator RBCs coated with anti-human IgG followed by centrifugation at an optimal speed. Results are determined by visual examination of the wells. A positive test occurs when indicator RBCs bind to antibodies attached to the immobilized platelets, forming a "carpet" of RBCs spread over the bottom of the well. In the absence of platelet antibodies, centrifugation forces indicator RBCs into a tight button at the well bottom.

SPRCA has been successfully used as a crossmatching test, because of its speed and ease of use.[14,37] Shortcomings include low specificity, subjective end point, short expiration of indicator cell reagent, and inability to determine HPA specificity of antibodies in the presence of non-HPA antibodies, e.g., HLA class I and ABO. Variations of this assay have been developed that use anti-human Ig-coated latex beads in place of indicator RBCs, which significantly extends the shelf life of the indicator reagent.[11]

The importance of including tests that use intact platelets, like flow cytometry and SPRCA, is that the antigenic epitopes recognized by platelet-reactive antibodies are better preserved on intact platelets. Some HPA-3 antigens can only be detected using intact platelets because HPA-3a and HPA-3b epitopes are labile in storage or apparently disrupted during detergent solubilization steps required when using antigen capture assays,[38,39] which are described in the next section.

Antigen-capture assays

Antigen-capture ELISA and modified antigen-capture ELISA

The principle of antigen-capture assays is to adhere individual platelet GPs, e.g., GPIIb/IIIa, to a solid support (polystyrene bead or microtiter plate well) for detection of platelet-specific antibodies (Fig. 2). GP captured from panels of platelets with different HPA phenotypes can be used to determine the specific HPA (e.g., HPA-1a) recognized by antibody present in serum samples.

The antigen-capture ELISA (ACE)[15] and modified

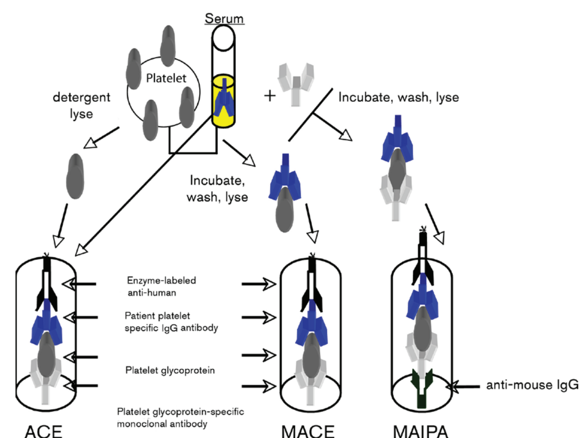

Fig. 2 Depiction of three different antigen-capture ELISAs for platelet antibody detection and identification: **left**, antigen-capture ELISA (ACE); **center**, modified ACE (MACE); **right**, monoclonal antibody immobilization of platelet antigens (MAIPA).

antigen-capture ELISA (MACE)[17] are two platelet antigen-capture assays developed in 1987 and 1989, respectively. In the ACE, a platelet GP-specific monoclonal antibody is adhered by simple electrostatic and hydrophobic interactions to the bottom of a polystyrene microtiter plate well. Platelet GPs are released from the cell membrane when solubilized in a nonionic detergent such as Triton X-100. The platelet lysate is then incubated with the immobilized monoclonal antibody to capture the specific GP. The well is then washed and incubated with a protein solution, most commonly 1 to 2 percent BSA in PBS, to block sites on the plastic not coated with antibody-GP complexes to prevent nonspecific binding of Ig from patient serum. Serum is added to the well and incubated for 30 to 60 minutes at either room temperature or 37°C. After multiple washes with buffer, IgG platelet antibodies in the serum that bind to specific HPA on the GP are detected with an enzyme-labeled anti-human IgG reagent after addition of enzyme substrate and measurement of the optical density in an ELISA plate reader (Fig. 2). In a similar technique, designated the immunobead assay, a polystyrene bead instead of a microtiter well is used.[18] The MACE is simply a modified version of the ACE in which platelets are first incubated with serum and washed before detergent lysis (Fig. 2). The MACE was developed to prevent false-positive reactions that can occur in the ACE caused by naturally occurring human anti-mouse IgG antibodies (HAMA).[40,41] HAMA are present at detectable levels in about 30 percent or more of human sera. HAMA recognize sugar epitopes present on the mouse monoclonal IgG antibodies used for GP capture.[41] Incubation of patient serum with platelets first, followed by washing, removes HAMA before addition of GP-antibody complexes to immobilized mouse monoclonal IgG. Dilution of patient serum in buffer containing 10 percent mouse serum is often used to inhibit interference from HAMA in the ACE.

The ACE assay not only is used for detection and identification of platelet alloantibodies, but also has been used successfully for detection of platelet autoantibodies in the serum of patients with ITP.[34] An acid eluate of the patient's washed platelets can be incubated with captured GPIIb/IIIa, GPIb/IX, and GPIa/IIa, the most common GP targets of platelet autoantibodies, as a direct test. Patient's serum can be incubated with the same targets for indirect testing. Elution of autoantibodies from the patient's platelets (breaks up immune complexes nonspecifically attached) and testing against platelet GPs significantly increases the specificity of the assay.[34]

Monoclonal antibody immobilization of platelet antigens

The monoclonal antibody immobilization of platelet antigens (MAIPA) assay was developed by Mueller-Eckhardt and colleagues,[16] also in 1987, and is more popular outside the United States. As in the MACE, platelets are first incubated with serum and washed, but then incubated with monoclonal antibody and washed again, followed by detergent lysis. GP-antibody complexes are captured from the lysate by anti-mouse IgG polyclonal antibodies adhered to the well of a microtiter plate (Fig. 2). A flow cytometry version of the MAIPA has been reported, in which a small bead instead of a microplate well was used as a solid support for monoclonal antibody capture of GP.[42]

Protein bead arrays

Antigen capture assays are being developed in which individual platelet GPs are attached to polystyrene microbeads.[43,44] Protein bead arrays of this nature were first developed and used for detection of antibodies to HLA class I and class II antigens.[45] These highly sensitive assays are now considered standard tests for HLA antibody detection and identification. The microbeads used have different bead "addresses," with different antigens attached to beads bearing variable amounts of flourescent dye incorporated into them at manufacture. For example, four individual purified platelet GPs can be attached to each of four separate beads containing different ratios of a green and a red fluorescent dye: GPIIb/IIIa on bead 1, GPIb/IX on bead 2, GPIa/IIa on bead 3, and GPIV on bead 4 (Fig. 3). The four beads are then incubated in the same well of a microtiter plate or test tube, together with patient's serum containing antibodies, for example, against GPIIb/IIIa. The beads are then washed and incubated with PE-labeled anti-human IgG. Fluorescence analysis of beads is performed on a Luminex instrument (Luminex Corp., Austin, TX). The Luminex is a small flow cytometer with two lasers: one (reporter) laser activates the fluorescent-labeled beads to identify their unique fluorescent addresses, and the other (detection) laser activates the PE-labeled anti-Ig for detection of antibody bound to GP on the bead. Each bead emits a specific intensity of light based on the amount of green fluorescence it contains (bead address), allowing for separation and identification of each of the four beads and simultaneous detection of PE fluorescence (patient antibody bound). Beads with PE fluorescence above an established threshold are interpreted as positive. Knowing which GP is attached to a specific, defined bead

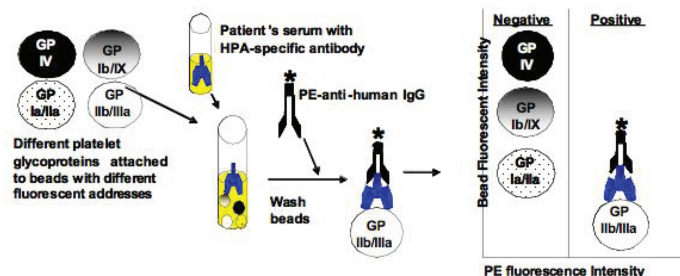

Fig. 3 Depiction of a protein bead array assay for platelet antibody detection and identification using microbeads coated with different platelet GPs, e.g., GPIIb/IIIa, GPIb/IX, GPIa/IIa, and GPIV. Patient's serum with antibody against GPIIb/IIIa reacts only with GPIIb/IIIa beads and is detected with PE-labeled anti-human IgG by fluorescence detection as depicted on the right side of the figure.

allows for the determination of GP and HPA specificity (Fig. 3). Modifications of this method have been reported using a standard flow cytometer and GPs attached to PE-labeled beads.[43] Protein bead arrays are highly sensitive and easily adapted to high-throughput applications because multiple antibodies with different HPA specificities can be detected by multiplexing all GP-coated beads in a single well of the plate. Issues with assay specificity have been observed in which antibodies are detected in sera from some normal, healthy people without previous history of alloimmunization. This reactivity is probably related to detection of antibodies in human sera that recognize neoantigens formed by conformational changes that occur in GPs when attached to the beads (authors' personal observations).

Recently, Fujiwara et al.[44] applied this technology in the form of the immunocomplex capture fluorescence analysis (ICFA) assay for detection of platelet and HLA class I antibodies, but instead used GP-specific monoclonal antibodies attached to the beads to capture antibody-GP immune complexes from detergent lysates prepared after incubating the patient's serum with platelets. In serum dilution studies, the ICFA showed fourfold higher sensitivity compared with the SPRCA for detection of anti-HPA5b and anti-GPIV, and for detection of HPA-3a antibodies compared with MAIPA, but lower sensitivity for detection of HPA-3a when compared with SPRCA.

Tests for heparin-dependent antibodies

Heparin-induced thrombocytopenia without (HIT) or with thrombosis (HITT) is a serious immune-mediated complication of heparin therapy characterized by (1) sustained thrombocytopenia that occurs during heparin therapy, (2) a platelet count that recovers after discontinuation of heparin, and (3) no other evident cause of thrombocytopenia.[46] HIT is caused by heparin-dependent antibodies (HDA) that target neoepitopes that form on PF4 owing to conformational changes induced by association with heparin. Immune complexes of PF4-heparin-HDA cross-link FcγRIIa receptors on platelets, causing platelet activation and release of additional PF4 from platelet alpha granules and formation of more PF4–unfractionated heparin (UFH)–HDA complexes. Sensitive laboratory tests can reliably confirm the diagnosis in patients suspected of having HIT.[47] Two types of tests are available: immunoassays and functional assays. Immunoassays detect HDA in patient sera that target the PF4 protein complexed with circulating heparin, whereas functional assays detect activation of normal target platelets after exposure to patient antibody in the presence of heparin. Several tests have been developed for detection of HDA including the PF4 ELISA, platelet aggregation, ^{14}C serotonin-release assay (SRA), and heparin-induced platelet activation (HIPA) assay. The three most frequently used of these assays (PF4 ELISA, SRA, platelet aggregation) will be discussed here.

PF4 ELISA

The PF4 ELISA was developed by Amiral et al.[48] after their discovery that PF4 complexed with heparin was the target for most HDA. This finding led to the production of several commercial kits for antibody detection. PF4 complexed with UFH or polyvinyl sulfate (PVS) is bound to the wells of a microtiter plate. Patient sera diluted from 1:50 to 1:100 are added to the wells, and after incubation and washing, an enzyme-labeled polyclonal anti-human IgG/A/M reagent is added for detection of HDA. Reactions with patient sera are typically considered positive if they exceed an optical density value of 0.400. Testing the patient's serum in parallel with 100 U/mL of added UFH to inhibit HDA reactivity can be performed to confirm positive results. Although debate still exists about the value of this added step,[49] it can help distinguish clinically significant positives from the many modest positive results seen with these highly sensitive assays in patients with a low clinical index for HIT.[50,51]

Another controversy is over the importance of detecting IgM and IgA antibodies in addition to IgG.[52] IgG HDA are thought to be more clinically significant because IgM and IgA HDA cannot crosslink and activate FcγRIIa receptors on platelets, which is why IgA and IgM HDA are not detected in the gold standard SRA. However, reports exist of patients with high clinical suspicion for HITT in which HDA of only the IgA or IgM class could be detected.[53,54] It is likely in rare cases that some strong IgM and IgA antibodies can cause HIT(T).

Platelet aggregation assay

The platelet aggregation assay using citrate platelet-rich plasma (PRP) has been in use for detection of HDA since the early 1970s and is commonly used today.[47] Platelet aggregation is evaluated in a standard platelet aggregometer using PRP incubated 1:1 with patient's citrate plasma and buffer or various concentrations of UFH (0.1–1.0 U/mL). Results are compared with those obtained with citrate plasma from a normal healthy donor. Performing the test in a lumi-aggregometer allowing for simultaneous monitoring of platelet activation and release of ATP may increase sensitivity of the method. Platelet aggregation is inexpensive and instrumentation is readily available; however, the test is time-consuming and lacks sensitivity and specificity compared with other assays.

SRA

The serotonin-release assay (SRA), first described by Sheridan et al.[55] in 1986, continues to be considered the gold standard assay for HDA. The SRA uses washed platelets. They are thought to increase sensitivity, because calcium can be added to optimize platelet activation, and to increase specificity, by removing plasma proteins that could cause unwanted platelet activation. ^{14}C-serotonin

is incubated with citrate PRP, which is taken up by dense granules inside the platelets. Platelets are washed once to remove plasma and residual radioactivity and suspended in Tyrode's buffer containing physiologic calcium. Labeled platelets, heat-inactivated test serum or citrate plasma, and 0.1 U/mL or 100 U/mL of UFH are incubated in wells of a microtiter plate with constant stirring. Sera with HDA activate platelets to release ^{14}C-serotonin. Platelets are pelleted, and released radioactivity is measured in the supernatant using a beta counter. Typically sera that release more than 20 percent of the radioactivity with 0.1 U/mL UFH and less than 20 percent at 100 U/mL UFH are considered positive for HDA. Testing performed in the presence of blocking monoclonal antibodies specific for FcγRIIa can also be performed to demonstrate inhibition of the reaction,[47] but this step does not confirm that the reaction is heparin-dependent, and could actually mask the presence of a non–heparin-dependent antibody, e.g., HLA class I, HPA, or platelet autoantibodies, if testing in the presence of 100 U/mL UFH is not also performed. Non-HDA can usually be differentiated from HDA because the former will not be inhibited in the presence of 100 U/mL UFH.

Despite being the gold standard, the SRA is a difficult assay to master. It also requires the use of relatively fresh platelets, good controls, and, of course, radioactivity.

Platelet Antigen Typing

Serologic typing methods

The platelet antibody detection methods previously described have been adapted for use in serologic typing of platelet antigens. However, serologic typing has declined dramatically in use because it is difficult and time-consuming to perform, and requires the availability of large volumes of highly specific anti-sera and the need to isolate sufficient platelets from whole blood.

Molecular typing methods

With the development of PCR and determination of the molecular basis of HPAs, serologic platelet antigen typing was largely replaced by DNA typing. This shift was a highly significant event in improving laboratory testing and advancing the ability to better diagnose alloimmune platelet disorders. Typing DNA for allelic variations that determine HPA was made possible with identification of the HPA-1a/1b (Pl$^{A1/A2}$) polymorphism in the gene encoding GPIIIa (βIII integrin).[19] This finding resulted in development of the first genotyping methods for HPA polymorphisms.[19,20] Since then, the explosion in molecular diagnostics during the last 15 years has resulted in the development of a variety of methods for platelet genotyping that are much improved in their speed and accuracy. It is beyond the scope of this article to describe every assay; therefore, we will describe some of the most common techniques in use today and direct readers to more detailed reviews of the subject.[56,57]

Allele-specific PCR (ASP)

Of the 21 biallelic platelet alloantigens described to date, 20 are the result of a single nucleotide polymorphism (SNP) in the genes that encode the proteins that express them (Table 1).[21,23] SNPs are single base changes in a gene that differentiate one allele from its antithetical allele. For example, if the base T is at position 196 of the gene integrin beta III (*ITGB3*) that encodes GPIIIa, then an individual expresses the HPA-1a form of GPIIIa; however, if T is replaced by a C at position 196 of *ITGB3*, then the person expresses the HPA-1b form of GPIIIa on his or her platelets. Taking advantage of this simple difference, primers complementary to short sequences that flank the 196 position of *ITGB3* are mixed with patient genomic DNA, individual deoxynucleotide triphosphates (dNTPs), *Taq* polymerase, and buffer containing an optimum concentration of Mg^{2+}. The mixture is subjected to 20 to 45 cycles of high-temperature denaturation of DNA to separate the double strands, followed by lowering the temperature to allow the primers to attach to (anneal) the separated strands. Thermostable *Taq* polymerase then extends the primers to copy (amplify) the stretch of DNA across the area containing the base at position 196. The resultant PCR amplicon is then separated out, usually on an agarose gel, stained with ethidium bromide, and exposed to UV light for visual fluorescence detection of the gel band (Fig. 4). If the DNA being genotyped contains a C instead of T at 196, then *Taq* cannot amplify the separated DNA strand with the primers complementary to DNA with a T at 196, and thus no PCR amplicon forms and no band appears in the gel.

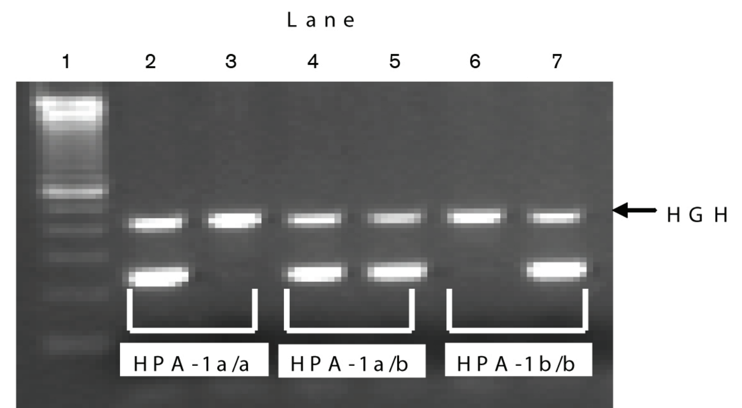

Fig. 4 Ultraviolet light transillumination results of ethidium bromide–stained 2 percent agarose electrophoresis gel showing separated allele-specific PCR products from genotyping assay for HPA-1. **Lane 1**: DNA size standards; **lanes 2, 4, 6**: HPA-1a allele typings; **lanes 3, 5, 7**: HPA-1b allele typings. One individual's HPA-1a/1b genotyping is shown for each pair of lanes: **lanes 2 and 3**, HPA-1a/1a; **lanes 4 and 5**, HPA-1a/1b; **lanes 6 and 7**, HPA-1b/1b. Human growth hormone (HGH) PCR control band is denoted by arrow.

Allele-specific PCR is relatively simple and inexpensive to perform and continues to be a popular method for HPA genotyping.[58] The only drawbacks of the method are requirements for post-PCR processing and subjective interpretation, which combined make typing slower and increase the potential for human error.

Melting curve analysis

Melting curve analysis (MCA) is a method to measure the dissociation (melting) attributes of double-stranded DNA (dsDNA) that occur during heating.[59,60] At higher temperatures, the two strands of dsDNA separate, and the heat required for the intrabase hydrogen bonds to be broken is dependent on their length, GC content, and complementarity. One popular method of MCA for platelet antigen genotyping uses the LightCycler instrument (Roche Diagnostics, Indianapolis, IN).[59,60] The assay begins with PCR amplification of patient DNA using primer pairs that flank the SNP of interest. As PCR product is formed, it is detected with fluorescent-labeled oligonucleotide probes that anneal to sequences adjacent to the SNP. One is a shorter allele-specific "reporter" probe and the other, a longer "anchor" probe. When both probes are bound, light from a diode activates the fluorescent labeled (usually fluorescein) anchor probe and the fluorescence resonance energy transfer (FRET) from the anchor probe to the reporter probe excites it to fluoresce. The fluorescence signal detected by the LightCycler is proportional to the amount of amplicon present, allowing for real-time monitoring and quantification. For MCA, the temperature is raised after PCR, and the shorter reporter probes, which bind to the amplicon with reduced stability, dissociate (melt) at lower temperatures, resulting in loss of fluorescence signal that can be monitored as a melting curve. The thermal melting point (T_m) of the reporter probes is sequence-specific, allowing for differentiation of two alleles, e.g., HPA-1a and HPA-1b. By taking the negative first derivative (–dF/dT) of the melting curve, melting temperature histograms for individual probes can be created for easy visual comparison and discrimination of homozygous wild-type and mutant and heterozygous genotypes.[57] The assay lends itself to multiplexing to increase throughput, and it is even possible to find new SNPs by recognizing changes in the architecture of melting curves (Fig. 5).

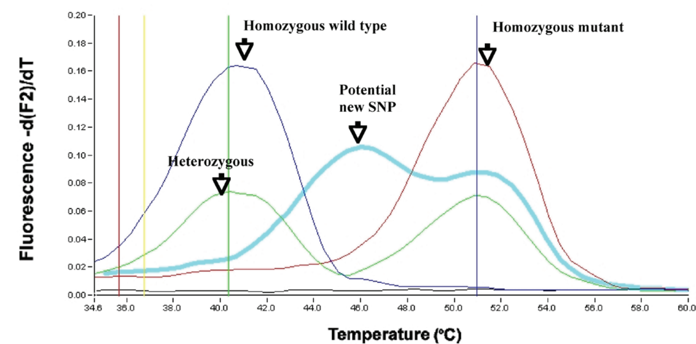

Fig. 5 Melting curve analysis by LightCycler for human platelet antigen (HPA) genotyping. Shorter reporter fluorescence probes bind with reduced stability to the DNA template containing the HPA SNP of interest, and as the temperature of the reaction is slowly raised they dissociate or "melt" off with resultant loss of fluorescence signal that displays as a melting curve. Each peak represents a different allele (e.g., HPA-3a **left peaks** and HPA-3b **right peaks**), which allows for genotype determination of homozygous wild-type, heterozygous, and homozygous mutant genotypes, as denoted by arrows. Potential new SNPs can be detected with this method as "hybrid" peaks that fall between the two individual allele peaks, as denoted in the figure.

5′-Nuclease or *Taq*Man assay

The 5′-nuclease or *Taq*Man assay is another popular genotyping method that also uses FRET chemistry.[61,62] The assay requires two sequence-specific probes, one that will hybridize with the DNA sequence containing the SNP for one allele (e.g., HPA-1a), and the other specific for the alternate allele (e.g., HPA-1b). Probes are labeled at their 5′ end with fluorochromes having different fluorescent emission spectra and a quencher molecule on their 3′ ends that prevents fluorochrome fluorescence as long as the probe is intact. Sequence-specific primers that flank the HPA SNP of interest are used to amplify the DNA by PCR. Probes and primers are added together with DNA for PCR, and as *Taq* polymerase amplifies and extends the primers, the 5′-nuclease activity inherent in *Taq* degrades the probe hybridized to the SNP-containing DNA sequence. This releases the 5′ fluorochrome, separating it from the quencher and allowing it to fluoresce, which generates a detectable, quantifiable signal. The allele-specific probe that is not complementary does not bind and prevents *Taq* from acting on the probe to release its fluorescence.

The 5′-nuclease and MCA methods lend themselves to moderately high throughput but are limited in their multiplexing capabilities by fluorochrome availability for probe labeling.

High-throughput platelet genotyping methods

Conventional PCR-ASP assays with gel end point for platelet genotyping have begun to give way to high-throughput methods. These methods are not only faster and higher volume, but they are less tedious to perform and they reduce human errors. Several methods using different chemistries and detection platforms have been reported, including glass slide and microplate arrays,[63] a liquid bead array,[64] and mass spectrometry.[65] Obstacles to high-throughput genotyping methods are the high costs of instrumentation and specialized reagents and the need for experienced individuals to implement, operate, and interpret the data. High-throughput genotyping platforms by

nature could have important applications, to screening all pregnant women for HPA-1a to identify those requiring antenatal treatment to prevent NATP, and for screening large numbers of blood donors to find matched platelet donors for highly alloimmunized patients.

Molecular tests for platelet genotyping are not without limitations. Rare cases have been reported of unknown SNPs located near the HPA polymorphism being typed that prevent proper annealing of ASP PCR primers and probes, causing false-negative results.[66] Silent HPA alleles and mutations that affect GP conformation, which produce discrepant genotype vs. phenotype results, have also been reported.[67,68] However, once identified these problems can be overcome, usually by redesign of primer and probe sequences.

Summary

Platelet antibody and antigen testing has advanced considerably since its inception with the use of first-generation assays that used the functional aspects of platelets for antibody and antigen detection. Introduction of the PSIFT assay paved the way for development of even more sophisticated flow cytometry immunofluorescence assays, solid-phase assays, and monoclonal antigen-capture assays. Antigen-capture assays such as the ACE, MACE, and MAIPA allowed for not only antibody detection but also identification of the specific HPA targeted by platelet antibodies and serologic typing. Identification of the HPA-1a/1b allelic polymorphism in the *ITB3* gene enabled development of ASP-PCR genotyping assays that quickly replaced serologic typing and greatly advanced the field of platelet immunology and laboratory diagnosis of immune platelet disorders. High-throughput platelet genotyping methods and protein bead arrays for platelet antibody detection and identification will further advance the field in the coming years.

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Brian R. Curtis, MS, MT(ASCP)SBB (corresponding author), and Janice G. McFarland, MD, BloodCenter of Wisconsin, Platelet & Neutrophil Immunology Laboratory, PO Box 2178, Milwaukee, WI 53201-2178.

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