

Platelet antigens and antibody detection

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Introduction

Platelets are anucleate blood cells, which play an important part in haemostasis and maintenance of the integrity of blood-vessel endothelium. Interaction of platelet glycoproteins (GPs) with extracellular matrix proteins is critical to this function.

The original impetus for investigating the alloantigens found on the GPs of the platelet membrane came from clinical observations of pathological conditions involving antibodies against platelet-specific antigens. Since then, dramatic progress has been made in elucidating the biochemical nature, function and molecular biology of platelet membrane GPs. The structure of many of the genes controlling platelet GPs have been determined and further characterization of platelet polymorphisms has been possible because of advances in molecular biology and monoclonal antibody techniques. These techniques have also resulted in a significant increase in the reliability of detection and identification of platelet antibodies and antigens in clinical laboratories.

Antigen nomenclature

The human platelet antigen (HPA) nomenclature system was adopted in 1990 to overcome problems with the previous nomenclature. Since then, more antigens have been described and meanwhile the molecular basis of many has been resolved. The nomenclature was revised again in 2003 (see further reading). To date, 24 platelet-specific alloantigens have been defined by immune sera, of which 12 are grouped into six biallelic systems (HPA-1, -2, -3, -4, -5 and -15). For the remaining 12, alloantibodies against the antigen but not the antithetical antigen have been observed. The molecular basis of 22 of the 24 serologically defined antigens has been resolved, and these antigens have been designated as HPA antigens (Table 1). In all but one of the 22, the difference between self and non-self is defined by a single amino-acid

substitution, caused by a single nucleotide polymorphism (SNP) in the gene encoding the relevant membrane GP (Table 2). For the six biallelic HPA systems, SNP typing on large numbers of DNA samples has provided reliable information on allele frequencies, with significant differences between populations.

Polymorphisms of the glycoprotein complexes

IIb/IIIa

The GPIIb/IIIa ($\beta_3\alpha_{IIb}$) complex is a heterodimeric integrin consisting of non-covalently associated α and β subunits, and acts as a receptor for fibrinogen, fibronectin, vitronectin and von Willebrand factor (VWF). The former three molecules are recognized via the Arg-Gly-Asp (RGD) tripeptide. There are approximately 50–80 000 copies of the heterodimer per platelet and it requires Ca^{2+} ions for its function. Upon platelet activation, GPIIb/IIIa undergoes a conformational change that permits fibrinogen to bind.

The genes coding for GPs IIb and IIIa are located on the long arm of chromosome 17 within a single 260-kb segment. The genetic basis of 11 alloantigens on the complex is known, 10 of which involve single nucleotide substitutions (Table 2). The HPA-14bw antigen is an exception in that it is the result of an 'in-frame' deletion of three nucleotides and that it has arisen from a mutation in the HPA-1b allele rather than the HPA-1a form or 'wild type'.

Linkage disequilibrium between certain HPA mutations has been described. There is linkage between the HPA-1b and 3b alleles; the HPA-3b allele is associated with a 9-bp deletion in intron 21 of the GPIIb gene, HPA-9bw is linked to the HPA-3b allele, and a rare Leu40Arg polymorphism on GPIIIa is linked to HPA-1b.

Ib/IX/V

The GPIb/IX/V complex (CD42) is involved in the initial stages of platelet adhesion at high shear-stress to damaged vessel wall via VWF in the subendothelial matrix. The VWF receptor is composed of four transmembrane components. GPIb α and GPIb β are covalently linked by a single disulphide bond, and are also non-covalently associated with the other two components, GPIX and GPV. All are members of the family

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Table 1 HPA antigens (reproduced with permission from *Vox Sanguinis* 2003; 85:240–245)

System	Antigen	Original names	Glycoprotein	CD			
HPA-1	HPA-1a	Zw ^a , Pl ^{A1}	GPIIIa	CD61			
	HPA-1b	Zw ^b , Pl ^{A2}					
HPA-2	HPA-2a	Ko ^b	GPIb α	CD42b			
	HPA-2b	Ko ^a , Sib ^a					
HPA-3	HPA-3a	Bak ^a , Lek ^a	GPIIb	CD41			
	HPA-3b	Bak ^b					
HPA-4	HPA-4a	Yuk ^b , Pen ^a	GPIIIa	CD61			
	HPA-4b	Yuk ^a , Pen ^b					
HPA-5	HPA-5a	Br ^b , Zav ^b	GPIa	CD49b			
	HPA-5b	Br ^a , Zav ^a , Hc ^a					
	HPA-6bw	Ca ^a , Tu ^a					
	HPA-7bw	Mo ^a					
	HPA-8bw	Sr ^a					
	HPA-9bw	Max ^a					
	HPA-10bw	La ^a					
	HPA-11bw	Gro ^a					
	HPA-12bw	Iy ^a					
	HPA-13bw	Sit ^a					
	HPA-14bw	Oe ^a					
	HPA-15	HPA-15a			Gov ^b		
		HPA-15b			Gov ^a		
		HPA-16bw			Duv ^a	GPIIIa	CD61

An online version of this table, an extended version of Table 2 and other tables detailing HPA alleles and platelet antigens without HPA assignment can be found at: <http://www.ebi.ac.uk/ipd/hpa/>

Table 2 Polymorphisms resulting in HPA antigens (reproduced with permission from *Vox Sanguinis* 2003; 85:240–245)

Antigen	Glycoprotein	HGNC	Chromosome	Nucleotide change	Amino acid change	
					Precursor	Mature protein
HPA-1	GPIIIa	<i>ITGB3</i>	17	176T > C	L59P	L33P
HPA-2	GPIba	<i>GP1BA</i>	17	482C > T	T161M	T145M
HPA-3	GPIIb	<i>ITGA2B</i>	17	2621T > G	I874S	I843S
HPA-4	GPIIIa	<i>ITGB3</i>	17	506G > A	R169Q	R143Q
HPA-5	GPIa	<i>ITGA2</i>	5	1600G > A	E534K	E505K
HPA-6w	GPIIIa	<i>ITGB3</i>	17	1544G > A	R515Q	R489Q
HPA-7w	GPIIIa	<i>ITGB3</i>	17	1297C > G	P433A	P407A
HPA-8w	GPIIIa	<i>ITGB3</i>	17	1984C > T	R662C	R636C
HPA-9w	GPIIb	<i>ITGA2B</i>	17	2602G > A	V868M	V837M
HPA-10w	GPIIIa	<i>ITGB3</i>	17	263G > A	R88Q	R62Q
HPA-11w	GPIIIa	<i>ITGB3</i>	17	1976G > A	R659H	R633H
HPA-12w	GPIbb	<i>GP1BB</i>	22	119G > A	G40E	G15E
HPA-13w	GPIa	<i>ITGA2</i>	5	2483C > T	T828M	T799M
HPA-14w	GPIIIa	<i>ITGB3</i>	17	1909–11delAAG	K637del	K611del
HPA-15	CD109	<i>CD109</i>	6	2108C > A	S703Y	S682Y
HPA-16w	GPIIIa	<i>ITGB3</i>	17	497C > T	T166I	T140I

HGNC, the name assigned to the encoding gene by The Human Genome Organization (HUGO) Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature>). Nucleotide change: nucleotide numbers are given in relation to the reference sequence in the NCBI database. These may be different to the numbers in the original publication describing the mutation. Nucleotide and protein substitutions are shown as changes from the more common form (a) to the less common form (b). In certain cases, e.g. HPA-5, the reference sequence encodes the less common form.

of leucine-rich repeat proteins. There are approximately 25 000 copies of GPIb/IX and 12 000 copies of GPV per platelet, and the whole complex is functionally associated with the low-affinity Fc receptor Fc γ RII (CD32). The primary VWF binding site has been localized within GPIb α , but there is evidence that the other three components also appear to contribute to receptor function.

The chromosomal localization of the relevant genes is known: the GPIb α gene is on chromosome 17, the GPIb β gene is on chromosome 22 and the GPIX and GPV genes are both on chromosome 3. Unusually, in each gene the majority of the protein-encoding sequence is present within a single exon, with one or two 5' non-translated exons. The structural similarity of the Ib-V-IX genes suggests that they may have evolved from a common ancestral gene. The genetic basis has been determined for two alloantigen systems present on the complex [Table 2]. The HPA-2 polymorphism is in linkage disequilibrium with the molecular-weight polymorphism of GPIb α . This weight polymorphism has no obvious functional consequences and results from variability in the number of 39-bp tandem repeats corresponding to 13 amino acids from Ser399 to Thr411 in the macroglycopeptide region of the GP. Sequencing of the GPIb α gene has revealed several silent mutations.

Ia/IIa

The GPIa/IIa (CD49/CD29, $\alpha_2\beta_1$) complex or VLA-2 (very late antigen) is another integrin and is found on activated T

lymphocytes and several other cell types. GPIa is the 165-kDa α_2 chain and GPIIa is the 145-kDa β_1 chain. The principal ligand of this $\alpha\beta$ heterodimer is collagen in exposed sub-endothelium. There are approximately 800–2800 copies of GPIa/IIa per platelet.

The genetic basis has been determined for both alloantigen systems present on GPIa/IIa [Table 2]. Two silent substitutions at position 807 and 873 of the GPIa gene have been observed to be associated with variable expression of the GP complex, and reports suggest that the 807 polymorphism and the HPA-5 polymorphism define three allelic forms of the gene. The HPA-13 mutation is unusual in that it alters the function of the GP, as platelets from HPA-13bw-positive individuals have a reduced response to collagen in aggregation studies and reduced spreading on a collagen surface.

Other antigen systems

The HPA-15 alloantigens are localized on CD109, a 175-kDa glycosylphosphatidylinositol (GPI)-linked GP found on platelets, monocytes, granulocytes, stimulated T cells and CD34-positive myeloid progenitor cells. Although the precise function of CD109 is unknown, recent reports suggest that it may be involved in cell-substrate and cell-cell interactions. The genetic basis for the HPA-15 antigens on CD109 has been determined (Table 2).

Two other alloantigen systems have been identified but the molecular basis has yet to be elucidated; Va^a is a low frequency antigen found on the GPIIb/IIIa complex and Mou^a is an antigen on a molecule still to be determined.

HPA typing

Accurate typing of patients for HPA antigens is required in several different clinical situations and blood services need to maintain panels of HPA-typed apheresis-platelet donors and whole-blood donors to support HPA-alloimmunized patients. The value of serological HPA phenotyping is limited as often too few platelets can be obtained from thrombocytopenic patients, and reliable serotyping reagents for HPA antigens, other than HPA-1a and HPA-5b, are rare. HPA antisera often contain human leucocyte antigens (HLA) class I antibodies, thus limiting their use to GP-specific assays such as the monoclonal immobilization of platelet antigen (MAIPA) assay. Monoclonal antibodies are now routinely used for red-cell phenotyping but, with the exception of HPA-1a, no monoclonal antibodies have been developed for HPA typing. However, several methods have recently been published for rapid screening assays using either polyclonal or recombinant anti-HPA-1a. These phenotyping assays can complement genotyping assays for the provision of HPA-selected donor panels.

In contrast, HPA genotyping can be carried out with genomic DNA obtained from any suitable cellular material

and commercially available reagents are used. The molecular basis of 22 platelet-specific antigens has been resolved and 21 of these are identified as a SNP. Many techniques have been described for SNP typing, several of which have been applied to HPA genotyping (see Suggested reading), although only a few are used widely. PCR with sequence-specific primers (PCR-SSP) has been adopted by many laboratories because of its simplicity, and protocols that allow the determination of several HPA genotypes under the same PCR conditions have been described.

Antibodies against platelet alloantigens

Clinical consequences of HPA alloantibody formation

Neonatal alloimmune thrombocytopenia

NAITP, often known as feto-maternal alloimmune thrombocytopenia (FMAIT), occurs as a consequence of maternal immunization against fetal platelet alloantigens inherited from the father. Maternal IgG alloantibodies then cross the placenta and cause immune destruction of platelets *in utero*, a situation analogous to haemolytic disease of the newborn, except that approximately 30% of cases occur in the first pregnancy.

NAITP, previously thought to be a rare disease, is the most common cause of severe thrombocytopenia in an otherwise healthy term neonate. A recent study of 25 000 pregnancies showed that approximately 1/350 pregnancies is complicated by anti-HPA-1a antibodies, and the incidence of severe thrombocytopenia due to anti-HPA-1a is 1/1200 live births.

There is wide variation in the clinical severity of the disease. Many cases have asymptomatic thrombocytopenia, but up to 10% of cases have intracranial haemorrhage and this can result in severe neurological damage or death. Subsequent pregnancies are usually affected, with similar or increasing severity. Treatment of severely affected cases is usually by transfusion of HPA-compatible platelets, either *in utero* or post delivery.

In whites, 80–90% of cases are due to anti-HPA-1a, 5–15% are due to anti-HPA-5b, and the remainder are caused by other HPA antibodies. Mothers may also be immunized against HLA antigens, but the involvement of HLA antibodies in NAITP has not been proven. At present, there is no routine antenatal screening, even though the incidence of the disease is higher than that of severe haemolytic disease of the newborn and several pilot studies have demonstrated that routine screening is feasible. However, introduction of screening needs to be based on a careful analysis of costs and benefits and the untoward effects of screening cannot be disregarded.

Post-transfusion purpura

PTP is a rare but severe disease that occurs approximately a week after transfusion of any blood product containing platelets or platelet membranes. A conservative estimation of

its incidence suggest that it occurs in 1/50 000–100 000 transfusions. The typical patient is a middle-aged or older woman, although PTP has occasionally been reported in men. All patients have had previous exposure to allogeneic platelets through either pregnancy or blood transfusion. Severe thrombocytopenia usually occurs within 5–10 days of the precipitating transfusion, and at the same time high-titre complement-fixing HPA alloantibodies can be detected in the patient's serum. The majority of cases involve anti-HPA-1a, although other specificities have been reported.

The pathogenesis is unknown; the patients are presumably sensitized by previous pregnancy or transfusion and respond to a second challenge of incompatible platelets by making high-titre HPA (and often HLA) antibodies. The resulting immune destruction of transfused platelets may contribute to the transfusion reactions that commonly occur but it does not explain the simultaneous destruction of the patient's own platelets, which are negative for the antigen concerned. Two mechanisms have been proposed. The destruction of transfused platelets may release alloantigen that is adsorbed onto the surface of the patient's own platelets, followed by attachment of alloantibody and consequent removal from the circulation. Alternatively, several groups have proposed the simultaneous but short-lived formation of autoantibodies; however, as yet no one mechanism has been shown to be operating in all cases of PTP.

PTP is a life-threatening disease as bleeding is often severe. Platelet transfusion is generally not recommended in PTP as both random and HPA compatible platelets are usually ineffective in achieving increments. Most patients respond to high-dose intravenous immunoglobulins, which is regarded as the most optimal first-line therapy. In the small number of patients who have been left untreated, spontaneous recovery usually occurs within 1–4 weeks of the onset of thrombocytopenia.

Refractoriness to platelet transfusion

This condition is defined as an inadequate increment in the platelet count following transfusion of random ABO-identical donor platelets. It is a common complication in patients receiving multiple platelet transfusions. Most cases of refractoriness have non-immune causes; however, a significant proportion (26–71%) of patients on long-term platelet transfusion will develop HLA antibodies. Approximately 10% of patients refractory to random donor platelets because of HLA antibodies also develop HPA antibodies; however, their relative contribution to refractoriness is difficult to determine. The specificity of HPA antibodies in this clinical setting has only been established in a few relatively small studies, but antibodies against the low frequency alloantigens HPA-1b, and -5b and -2b appear to be the most common.

Methods used for the detection of platelet-specific antibodies

Although a wide range of techniques have been developed for HPA antibody detection over the last 25 years, four techniques, or modifications of them, have become the most common; (1) the platelet immunofluorescence test (PIFT); (2) monoclonal antibody immobilization of platelet antigens (MAIPA) assay; (3) the solid-phase red cell adherence assay; and (4) a variety of ELISA-based techniques.

The PIFT, described by von dem Borne *et al.* in 1978, was the first simple, reliable and versatile method for the detection of platelet-reactive antibodies and it stimulated interest in platelet immunology. The MAIPA assay, described by Kiefel *et al.* in 1987, was equally important because of its sensitivity and ability to differentiate between mixtures of antibodies. It is this technique (Fig. 1) that has led to the discovery of many new platelet antigens in recent years.

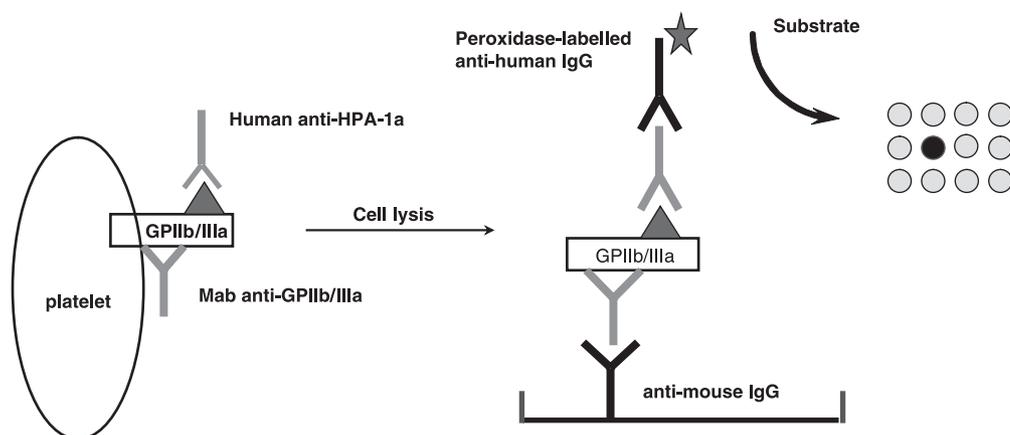


Fig. 1 Detection of HPA antibodies by MAIPA assay. Intact washed platelets are first incubated with human serum (containing anti-HPA-1a in this example) and then a monoclonal antibody against the glycoprotein under investigation (GPIIb/IIIa in this example). Platelets are then lysed and the supernatant is added to a microplate precoated with antimouse IgG. Any human antibodies bound to the glycoprotein are detected with a peroxidase-labelled antihuman IgG and a suitable substrate.

Proficiency in antibody detection depends on a variety of factors, including techniques, cell panels and operator experience. A study of interlaboratory variation in platelet antibody detection showed that the best performers used at least two techniques, including both PIFT and MAIPA assay, and an HPA-genotyped cell panel. Consensus methods for PIFT and MAIPA assays are available on the internet site <http://www.nibsc.ac.uk/divisions/Haem/>.

Monoclonal antibodies against HPA antigens

Few monoclonal antibodies against HPA antigens have been produced. Some mouse monoclonal antibodies have been made against synthetic peptides containing the HPA-1a and -1b sequences using conventional hybridoma techniques. However, these antibodies do not react with the native GP on platelets due to the conformational nature of the antigen resulting from the amino-acid substitution. More recently, phage-display technology has been used by several groups to produce human monoclonal antibody Fv or Fab' fragments, which bind to the HPA-1a form of GPIIIa on intact platelets. Such antibodies have both diagnostic and potential therapeutic applications.

Other putative clinical consequences of HPA polymorphisms

Until recently the only clinical relevance of the HPA polymorphisms was their capacity to induce an immune response leading to the production of alloantibodies, as described above. The same substitutions might possibly also affect the function of the platelet GPs by altering their ligand-binding ability or the subsequent molecular events.

Platelet aggregates are found in coronary artery atherosclerotic plaques and are thought to be involved in the pathogenesis of myocardial infarction (MI). A study by Weiss *et al.* found a higher incidence of HPA-1b in patients with MI or unstable angina than in controls. However, although other groups quickly confirmed these results, some large-scale studies did not support the role of the HPA-1b polymorphism as an inherited risk factor for arterial thrombotic disease.

Some studies have investigated the incidence of HPA-1b in patients with stroke or venous thrombosis, diseases with similar pathogenesis, but there was no correlation with disease. Similarly, the HPA-2, -3 and -5 antigens were not correlated with an increased risk for stroke.

A similarly contradictory situation exists when *in vitro* data are considered. While most studies have failed to demonstrate any functional difference between HPA-1a1a and HPA-1b1b platelets, one study has shown a decrease in fibrinogen binding by HPA-1b1b platelets while another has shown an increase.

Suggested reading

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