FINNISH RED CROSS BLOOD TRANSFUSION SERVICE
AND DEPARTMENT OF MEDICINE,
UNIVERSITY OF HELSINKI, FINLAND

AUTOIMMUNE THROMBOCYTOPENIA

Detection of platelet-associated IgG, reticulated platelets and platelet Fcγ receptor polymorphism in thrombocytopenic patients

Lotta Joutsi-Korhonen

ACADEMIC DISSERTATION
To be publicly discussed with permission of the Faculty of Medicine, University of Helsinki, in the Nevanlinna-auditorium of the Finnish Red Cross Blood Transfusion Service, Kivihaantie 7, Helsinki, on the 26th of August 2000, at noon

Helsinki 2000

ACADEMIC DISSERTATIONS FROM THE FINNISH RED CROSS BLOOD TRANSFUSION SERVICE NUMBER 43

Supervisor

Docent Riitta Kekomäki, M.D.
Finnish Red Cross Blood Transfusion Service
Helsinki, Finland

Reviewers

Docent Erkki Elonen, M.D.
Department of Medicine
Helsinki University Central Hospital
Helsinki, Finland

Docent Eeva-Riitta Savolainen, M.D.
Department of Clinical Chemistry
University of Oulu
Oulu, Finland

Opponent

Docent Allan Rajamäki, M.D.
Department of Haematology
Central Laboratory
Turku University Central Hospital
Turku, Finland

ISBN 951-97576-6-X (nid.)
ISBN 951-97576-7-8 (pdf)
ISSN 1236-0341
Helsinki 2000
Yliopistopaino

Vita brevis,
ars longa,
occasio praeceps,
experimentia fallax,
iudicium difficile

To my husband Timo

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-IV).

I **Joutsi L,** Kekomäki R. Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG. Br J Haematol 1997;96:204-9.

II **Joutsi-Korhonen L,** Javela K, Hormila P, Kekomäki R. Glycoprotein V-specific platelet-associated antibodies in thrombocytopenic patients. Submitted

III **Joutsi L,** Javela K, Partanen J, Kekomäki R. Genetic polymorphism H131R of Fcγ receptor type IIA (FcγRIIA) in a healthy Finnish population and in patients with or without platelet-associated IgG. Eur J Haematol 1998;61:183-9.

IV **Joutsi-Korhonen L,** Sainio S, Riikonen S, Javela K, Teramo K, Kekomäki R. Detection of reticulated platelets: Estimating the fluorescence of thiazole orange stained platelets. Eur J Haematol 2000, in press.

In addition, some unpublished data are presented.

ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
CI	confidence interval
CV	coefficient of variation
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FcγR	receptor for Fc fragment of IgG
FcR	Fc receptor
FL	fluorescence
FSC	forward light scatter
GP	glycoprotein
HIT	heparin-induced thrombocytopenia
HLA	human leukocyte antigen
HPA	human platelet antigen
H131	histidine 131 allele of FcγR type IIA
Ig	immunoglobulin
kDa	kilodalton
ITP	idiopathic thrombocytopenic purpura
PA-IgG	platelet-associated IgG
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIFT	platelet immunofluorescence test
MACE	modified antigen capture ELISA
MAIPA	monoclonal antibody-specific immobilisation of platelet antigens
RNA	ribonucleic acid
RNAase	ribonuclease
RT	room temperature
RP	reticulated platelets
R131	arginine 131 allele of FcγR type IIA
SD	standard deviation
SLE	systemic lupus erythematosus
TBS	Tris-buffered saline
TO	thiazole orange

Amino acids and nucleotides are abbreviated to one-letter codes.

REVIEW OF THE LITERATURE

1. PLATELET MEMBRANE GLYCOPROTEINS AND FCγ RECEPTORS

1.1. Introduction to platelet physiology

Platelets prevent and arrest haemorrhage from blood vessel wall defects. The complex haemostatic system comprises the interactions of the vessel wall and of various plasma proteins as well as the platelets. Platelet membrane glycoproteins (GP) function as physiological receptors and mediate interactions between the vascular subendothelium and the platelets (adhesion) and platelet-platelet interaction (aggregation) as platelets respond to thrombogenic stimuli.

Platelets are produced by bone marrow megakaryocytes. A single mature megakaryocyte is suggested to ultimately produce several thousand platelets. Normally, the megakaryocytes give rise to nearly 2 x 10^{11} platelets daily (Majerus, 1994). Megakaryopoiesis and thrombopoiesis are regulated by cytokines, such as thrombopoietin (Kaushansky, 1998). Patients with depressed platelet production are reported to have higher thrombopoietin levels than patients in whom peripheral platelet destruction is increased (Emmons et al, 1996; Porcelijn et al, 1998). Moreover, the thrombopoietin levels of patients with increased peripheral platelet destruction seems to differ only slightly from those of healthy controls (Ichikawa et al, 1996; Kosugi et al, 1996a). In different conditions, the bone marrow may increase platelet production approximately five- to ten-fold, thereby maintaining the normal platelet count (Majerus, 1994). Normally, two thirds of the total platelet mass is circulating at concentrations of 150 to 400 x 10^9/l, the rest remains in the splenic circulation.

Platelets are anucleate cells with diameters of 2 to 4 μm, and a cell volume of approximately 10 fl (Majerus, 1994). Platelets change, upon activation, from a disc shape to a compact sphere with long extensions facilitating adhesion (Fig 1). The change in shape and retraction of the clot are mediated by the cytoplasmic actin and myosin. In addition to other cell organelles, such as peroxisomes, mitochondria and lysosomes, platelets contain dense granules and α-granules which secrete various substances and the proteins needed in platelet aggregation and coagulation reactions.

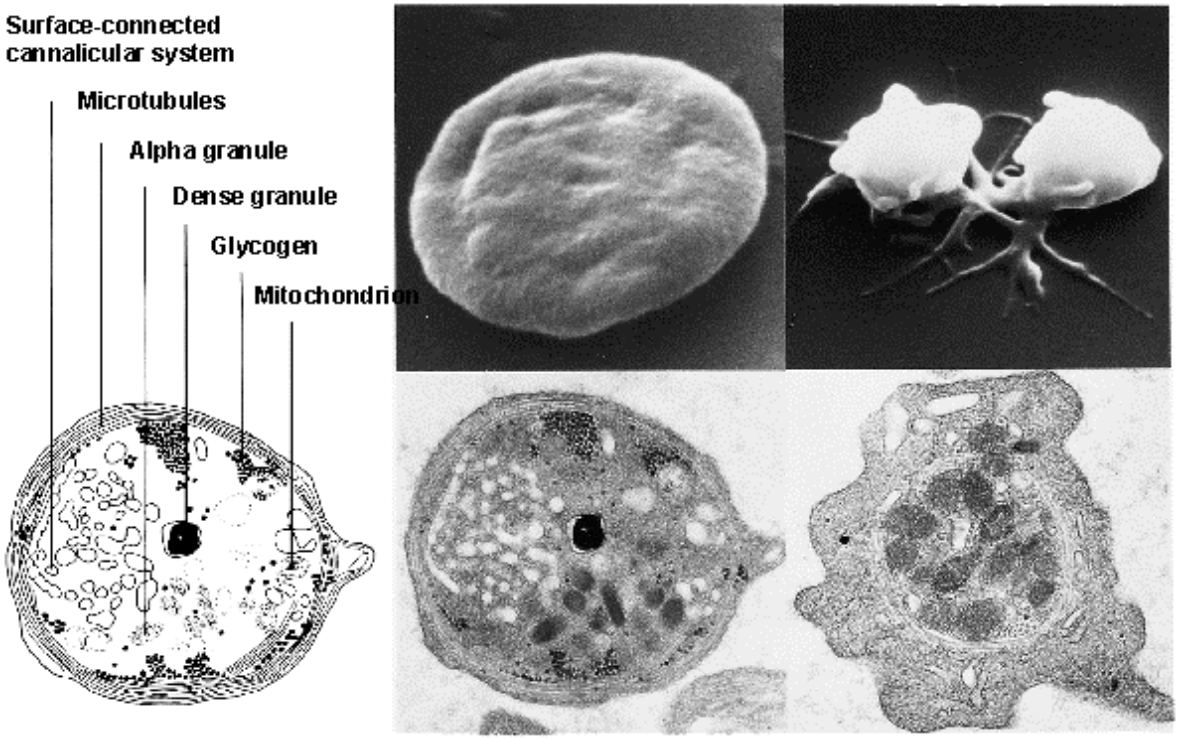

Fig 1. Electron micrograph of resting and activated platelets. Published with the permission of The Lancet: George JN. Platelets. Lancet 2000;355:1531-9.

Platelets have been demonstrated to vary in volume and density (Corash et al, 1977; Ault, 1993; Jackson & Carter, 1993). In general, platelet morphology may be studied from a peripheral blood smear, and the size distribution of the circulating platelets may be estimated with automated cell counters or flow cytometry. Some studies have shown that, in normal conditions, the platelet volume and density are unrelated to platelet age (Boneu et al, 1977; Mezzano et al, 1981). After acute bleeding, however, newly synthesised platelets are larger and have a higher density and increased ribonucleic acid (RNA) content (Ingram & Coopersmith, 1969; Ault & Knowles, 1995). In cases of intensive peripheral platelet destruction, the megakaryocytic cytoplasm and nuclear deoxyribonucleic acid (DNA) increase in quantity and the megakaryocytes seem to produce a platelet population with a greater mean and greater dispersion of volume (Boneu et al, 1977; Martin & Trowbridge, 1983). Moreover, the mean platelet volume in bone marrow blood has been shown to be higher than that observed in peripheral blood, suggesting that the newly formed cell population consists of larger platelets (Aliberti et al, 1996). The heterogeneity of platelet size can also be explained partly by

platelet microparticles, which are platelet-derived vesicles too small to be detected in routine platelet counting. The levels of the microparticles have been shown to be higher in patients with increased peripheral platelet destruction (Nomura et al, 1991; Jy et al, 1992). The significance of platelet microparticles still remains unknown, and the heterogeneity of platelet size and the characteristics of newly synthesised platelets are still of wide interest.

Investigation of platelet survival and determination of platelet sequestration are used for measurements of platelet kinetics. Platelet lifespan measurements may be performed by monitoring labelled and transfused platelets. Different radioisotopes, especially Indium-111, have been used as labels (International Committee for Standardisation in Hematology Panel on Diagnostic Applications of Radionuclides, 1988). However, isolation and labelling of platelets with radioisotopes form obstacles. Non-isotopic methods have also been applied for measurements of platelet lifespan, such as inhibition of monoamine oxidase or cyclo-oxygenase enzymes as well as biotinylation of platelets (Ault & Knowles, 1995; Dale, 1997). In general, however, platelet survival studies are considered to be complicated and to suffer from the requirement of several procedures, and therefore, they are rarely used clinically.

Normally, platelets with a mean lifespan of 7 to 10 days are destroyed in the spleen. The liver has also been suggested to be an important site of platelet destruction, especially in patients with increased peripheral platelet destruction (Aster & Jandl, 1964; Panzer et al, 1986; Najean et al, 1997). Accessory spleens may also maintain increased platelet destruction. In conditions with increased peripheral platelet destruction, the survival of cells ranges from a few days to a matter of minutes. For studies of the pathophysiological mechanisms of thrombocytopenia more practical measurements of platelet kinetics have proved necessary. Quantification of newly formed platelets would be useful and more easily adapted for clinical purposes and thus have advantages over platelet survival studies. In the circulation platelets with a high nucleic acid content, reticulated platelets (RP), have been thought to represent young platelets, and thus measurement of RP may give an estimate of megakaryocyte stimulation and platelet production (Ingram & Coopersmith, 1969; Kienast & Schmitz, 1990; Ault et al, 1992; Richards & Baglin, 1995).

1.2. Platelet membrane glycoproteins (GP)

Characteristics of the major platelet GPs are presented in Table 1. Target antigens of platelet autoantibodies have most frequently been studied in the GP IIbIIIa and GP IbIX complexes (van Leeuwen et al, 1982; Beardsley et al, 1984; Woods et al, 1984a & b; McMillan et al, 1987; Tomiyama et al, 1987 & 1989; Berchtold et al, 1989a; Kokawa et al, 1993). GP IIbIIIa and GP IbIX have been recognised to be the most immunogenic of the platelet glycoproteins, carrying some recognised autoepitopes, as well as the known alloantigens, clinically important polymorphisms (Kunicki & Newman, 1992; Ouwehand & Navarrete, 2000; Table 1). The Human Platelet Antigen (HPA) system is used as the nomenclature for the platelet-specific alloantigens (http://www.nibsc.ac.uk/Haem).

1.2.1. GP IIbIIIa

GP IIb and GP IIIa are among the major GPs in the platelet plasma membrane (Fig 2). The GP IIbIIIa complex is a member of the superfamily of integrins, consisting of α and β heterodimers ($\alpha_{IIb}\beta_3$; Table 1). The GP IIbIIIa complex is required for normal platelet aggregation. This complex is capable of binding not only to fibrinogen, but also to other adhesive proteins, such as fibronectin, vitronectin and von Willebrand factor. The GP IIbIIIa complex appears very early at the promegakaryoblastic stage, and it is considered the earliest marker of the megakaryocytic lineage. Upon activation, conformational changes and clustering of GP IIbIIIa have been demonstrated.

The calcium-dependent heterodimeric GP IIbIIIa complex is the most abundant glycoprotein on the platelet surface; 45 000 to 50 000 copies of GP IIbIIIa are exposed on each resting platelet (Table 1). The complex has been recognised to be the most immunogenic of the platelet glycoproteins, carrying most of the known alloantigens (Table 1). GP IIIa is the carrier of most of the HPAs described so far (Table 1). The genes for GP IIb and IIIa have been localised to chromosome 17. The gene for GP IIb spans a 22 kilobase length of DNA, whereas the gene for IIIa is larger, containing 14 exons and spans over 60 kilobases (Prandini et al, 1988; Lanza et al, 1990). GP IIb is composed of two disulphide-linked polypeptide chains: IIbα and transmembrane IIbβ, and the GP IIIa is a single-chain transmembrane polypeptide with multiple interchain disulphide bonds. Total absence or defective cell surface expression of platelet GP IIbIIIa is characteristic of Glanzmann´s thrombasthenia with impaired platelet aggregation and adhesion.

Table 1. Platelet membrane glycoproteins (GP) and their physical properties.

GP (subunits)	CD	Molecular weight†	HPA	Gene family	Complex	Ligand for the complex	Autoepitopic regions**	Platelet function	Molecules on resting platelet	Other cells expressing GP
IIb (α)	41	125	3, 9w, Va*	integrin	IIbIIIa	fibrinogen, vWf,	1) 65 kDa carboxyterm.	aggregation,	45 000	megakaryocytes
IIb (β)		23		integrin		fibronectin, vitronectin	1) 2E7, aa 231-238	adhesion at high shear	45 000	megakaryocytes
IIIa	61	95	1, 4, 6w, 7w, 8w, 10w, 11w, Oe*	integrin			1) 33 kDa cysteine-rich 2) cytoplasmic carboxyterminal 3) aa 734-739 4) IIbIIIa complex	rates	45 000	megakaryocytes, endothelium, fibroblasts, smooth muscle
Ib (α)	42b	141	2, Pe*	leucine-rich	IbIXV	vWf, thrombin	1) close to membrane 2) extracellular epitopes	adhesion	25 000	megakaryocytes, endothelium
Ib (β)	42c	22	12w	leucine-rich					25 000	
IX	42a	20		leucine-rich					25 000	
V	42d	80	Plt*	leucine-rich				(?)	11 000	
Ia	49b	155	5, 13w	integrin	IaIIa	collagen		adhesion, aggregation	††	activated T lymphocytes, endothelium, other cell types
IIa	29	138		integrin					10 000	endothelium
IV=IIIb	36	97	Vis*	(-)		thrombospondin, collagen		adhesion	15 000	monocytes, endothelium, erythroblasts
VI		67		Ig-domain		collagen		activation	?	
FcγRIIA	32	40		Ig-domain		Fc fragment of IgG		immune contact	600-8000	neutrophils, eosinophils, basophils, monocytes, macrophages
GMP-140	62	140		selectin		oligosaccharides of leucocytes		cellular interactions	(-)	endothelium

* Private platelet alloantigens in the complex. † Molecular weight of unreduced protein in kDa. ** Autoepitopes recognised by circulating antibodies. †† IaIIa complex: 800-3500/platelet.

aa: amino acids; CD: cluster of differentiation; GMP: granule membrane protein; HPA: human platelet antigen, Ig: immunoglobulin; vWf: von Willebrand factor.

Ref: Szatkowski et al, 1986; Tsubakio et al, 1987; Tomiyama et al, 1989; Kunicki et al, 1990, 1991a & b; Fujisawa et al, 1991 & 1992; Kekomäki et al, 1991; Kunicki & Newman, 1992; He et al, 1995; Bowditch et al, 1996.

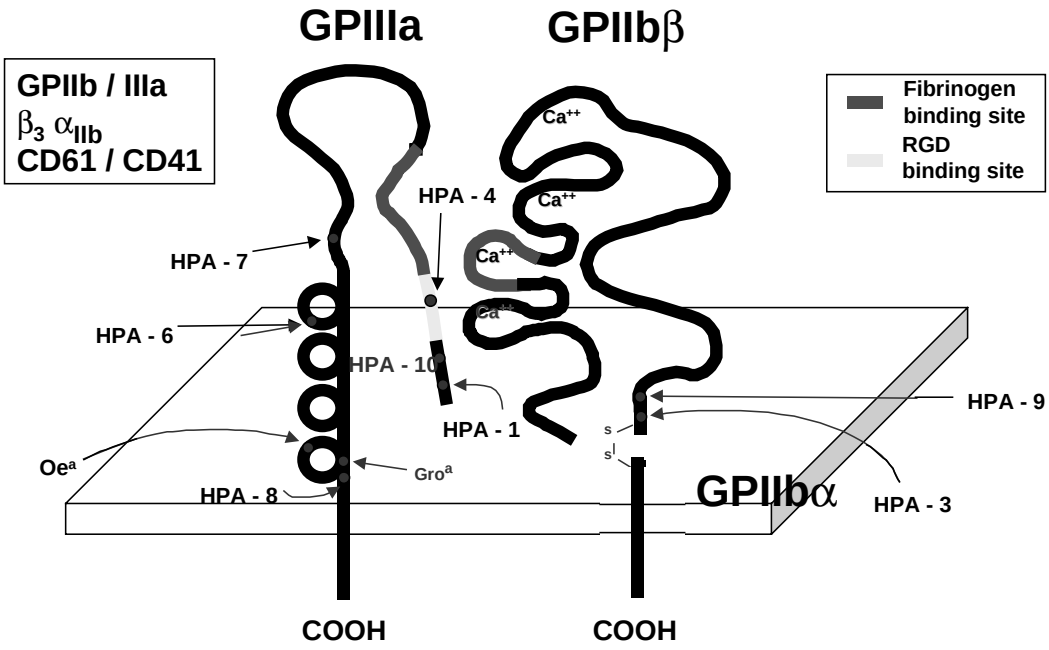

Fig 2. Platelet glycoprotein complex GP IIbIIIa. Human platelet antigens (HPA) are indicated. (Modified from http://www.nibsc.ac.uk/Haem, published with the permission.)

1.2.2. GP IbIXV

GP IbIX is involved in the adhesion of platelets to the exposed vascular subendothelium. In the circulation, with its high shear stress rates, this complex functions as a receptor for von Willebrand factor. The binding of von Willebrand factor to platelet GP IbIX causes platelet activation, which promotes the recruitment of additional platelets. Probably, GP Ib also plays a role in thrombin activation, since it appears to contain a binding site for thrombin. The GP IbIX complex also serves as a site of attachment for the cytoskeleton to the plasma membrane.

The leucine-rich proteins GP Ib and GP IX form a non-covalent, functional complex, and are integral membrane proteins spanning the lipid bilayer (Fig 3). The GP Ib has two disulphide-linked subunits, Ibα and β. The major and extramembranous portion of GP Ibα is called glycocalicin. The glycocalicin concentration in plasma has been suggested to reflect platelet turnover and platelet membrane injury in vivo (Beer et al, 1994). Yet the method for measuring glycocalicin has not been standardised, since methodological difficulties have limited its clinical applications. GP IX and GP V are composed of single

polypeptide chains, which form a non-covalent complex in the platelet membrane (Modderman et al, 1992; Fig 3). The genes for GP Ibα and GP Ibβ are located in chromosomes 17 and 22, respectively, whereas the genes for GP IX and GP V are located in chromosome 3. The genes for the GP IbIXV complex are relatively small, only a few kilobases, which is partly due to their few introns (Wenger et al, 1988; Yagi et al, 1995). In systematic screening, nine polymorphic sites have been found in the GP V gene, four of them leading to an amino acid change in the protein (Koskela, 1999).

GP Ibα, in addition to its HPA-2 polymorphism, has also been demonstrated to carry a length polymorphism and several silent polymorphism sites (Koskela, 1999). Defects in the GP IbIXV complex lead to impaired platelet function and diminished platelet survival in the autosomally recessively inherited macrothrombocytopenia known as Bernard-Soulier syndrome (López et al, 1998; Koskela, 1999).

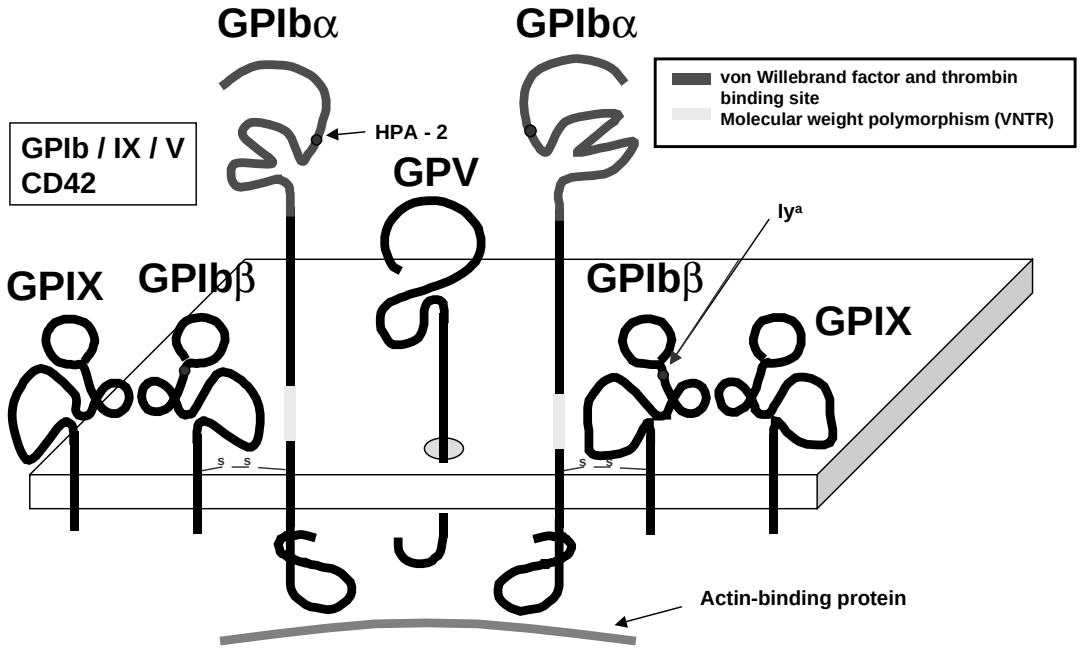

Fig 3. Platelet glycoprotein complex GP IbIXV. Human platelet antigens (HPA) are indicated. (Modified from http://www.nibsc.ac.uk/Haem, published with the permission.)

1.2.3. GP IaIIa

GP IaIIa is another member of the integrin superfamily of adhesion receptors ($\alpha_2\beta_1$). At low shear rates, the GP IaIIa complex functions as a receptor for collagen, and thus mediates a significant portion of the adherence of platelets to the subendothelium.

GP IaIIa consists of two unique subunits: α_2 (Ia) and β_1 (IIa), which are composed of single polypeptide chains containing numerous intra-chain disulphide bonds. GP Ia, which contains a transmembrane domain and a short cytoplasmic segment, is widely expressed by a variety of cells (Table 1). GP IIa is composed of a short cytoplasmic domain, which may vary in length because of alternative splicing. The genes for GP Ia and GP IIa are located in chromosome 5 and they contain a single exon and seven exons, respectively (Takada & Hemler, 1989). Two alloantigen systems have been reported on GP Ia, of which HPA-5 has been demonstrated to be almost as important, clinically, as HPA-1.

1.3. Introduction to Fcγ receptors

Receptors for the Fc fragment of IgG (Fcγ receptors, FcγR) are considered to be a contact link in the immune system. These proteins mediate contact between phagocytes and IgG-opsonised particles or cells. Phagocytes, monocytes, macrophages, neutrophils, eosinophils and also platelets need FcγRs for binding, ingestion and destruction of the opsonised particles. FcγRs on macrophages mediate the recognition and binding of IgG-coated cells in the mononuclear-phagocyte system. Triggering of FcγRs induces activation of phagocytes and leads to release of inflammatory mediators.

Three different subclasses of FcγRs have been identified: FcγR type I (FcγRI), FcγRII and FcγRIII (McKenzie & Schreiber, 1994; Kimberley et al, 1995). The Fcγ receptor subclasses have been demonstrated to be polymorphic and also to show different antibody binding affinities. Different cell types express the different subclasses (Table 2). FcγRIA is the only FcγR capable of binding monomeric IgG with high affinity. The three FcγRII genes, FcγRIIA, FcγRIIB and FcγRIIC, encode for proteins that bind weakly to monomeric IgG but more strongly to the IgG in aggregates. FcγRI is the least polymorphic of all the FcγRs, but both the FcγRIII genes, FcγRIIIA and FcγRIIIB, have been shown to be polymorphic (Table 2). FcγRIIIA has a functionally important polymorphic site in amino acid 158 (V158F). FcγRIIIB bears an important polymorphism of the neutrophil antigen (NA1/NA2) and a polymorphic site in amino

acid 266 (SH266), which both have unknown structural and functional implications (Lehrnbecher et al, 1999). FcγRIIIB is the most abundant FcR on neutrophils. However, the significance of the variety of FcγR subclasses related to genetic polymorphism, protein expression and ligand binding affinities remains an interesting but poorly understood matter.

Table 2. Fcγ receptors (FcγR), receptors specific for Fc fragment of IgG

	FcγRI	FcγRII	FcγRIII
CD	64	32	16
Gene transcripts*	IA IB1, IB2 IC	IIA IIB1, IIB2, IIB3 IIC	IIIA IIIB
Molecular weight	72 kDa	40 kDa	50 - 80 kDa
Affinity for ligand	High	Low	IIIA: medium; IIIB: low
Expression at the protein level	monocytes, macrophages, neutrophils†	IIA: neutrophils, basophils, eosinophils, monocytes, macrophages, platelets IIB: B cells	IIIA: macrophages, monocytes, NK cells IIIB: neutrophils, eosinophils†
Polymorphism (amino acid)		IIA: QW27 IIA: HR131 IIA: QK127	IIIA: LRH48 IIIA: VF158 IIIB: NA1/NA2 (36, 65, 82,106) IIIB: null type IIIB: SH266

* FcγR-encoding genes are all located on the q region of chromosome 1. † in contact with cytokines.
CD: cluster of differentiation; NA: neutrophil antigen; NK cells: natural killer cells.
References: McKenzie & Schreiber, 1994; Anderson et al, 1995; Kimberley et al, 1995; Koene et al, 1997; Norris et al, 1998; Lehrnbecher et al, 1999

The most widely expressed of all the FcγRs is FcγRII. The only FcγR expressed on platelets is FcγRIIA (Rosenfeld et al, 1985). FcγRII is an integral membrane glycoprotein of 40 kDa with two extracellular Ig-like domains, which is found on phagocytes, neutrophils, monocytes and macrophages and also at low levels on B cells. In addition, there is a soluble form of the protein, which may alternatively be spliced, lacking the transmembrane-encoding exon (Markovic et al, 1995). The FcγRIIA gene contains eight exons. At present, three polymorphic FcγRIIA genes are known to result in changes in amino acid positions 27 (Q27W), 127 (Q127K) and 131 (H131R).

2. THROMBOCYTOPENIA

2.1. Introduction to thrombocytopenia

The most common cause of abnormal bleeding is thrombocytopenia, defined as a subnormal platelet concentration in the circulating blood. The aetiology of thrombocytopenia may involve three different mechanisms: decreased platelet production, sequestration of cells or increased peripheral destruction of platelets.

Decreased platelet production may be due to hypoplasia or suppression of megakaryocytes. Bone marrow injury may be the consequence, for example, of neoplasias, myelosuppressive drugs, irradiation, aplastic anaemia or viral infections. Ineffective or insufficient platelet production or defects in the regulation of thrombopoiesis may also lead to decreased platelet production. Various disorders of the spleen may cause abnormal sequestration of platelets. Increased peripheral platelet destruction may be due to numerous conditions, and it commonly involves immunological processes. Autoimmune-mediated thrombocytopenia is more frequently encountered than the alloimmune condition. Platelet utilisation in intravascular thrombi or on damaged endothelial surfaces, such as are present in disseminated intravascular coagulation, is considered to be a non-immunological process leading to thrombocytopenia.

In laboratory conditions, pseudothrombocytopenia due to platelet clumping may cause falsely low platelet counts. Its prevalence is estimated to be 0.1% of routine blood counts. Pseudothrombocytopenia may be diagnosed by examining the peripheral blood smear (Berkman et al, 1991; Silvestri et al, 1995). A low platelet count in vitro, has been suggested to be due to the presence of agglutinating anticoagulant-dependent antibodies. Agglutination has been reported in blood anticoagulated with EDTA, citrate, oxalate or heparin. Such anticoagulant-induced thrombocytopenia should be recognised in order to avoid unnecessary investigations and clinical consequences. Thus, all significantly low platelet counts should be confirmed by a blood smear sample.

2.2. Autoimmune thrombocytopenia

2.2.1. Autoimmune disease

Autoimmune diseases occur when self antigens are recognised as foreign entities, and they may be either organ-specific or systemic disorders. It is agreed that genetic susceptibility, as well as potential environmental agents, influences the aetiological course of an autoimmune disease. In general, the induction of autoimmune disease probably involves T lymphocytes: autoimmune responses usually begin with the activation of CD4+ T helper cells. The number of CD8+ T cells has then been demonstrated to be increased; thus the CD4/CD8 T cell ratio is decreased. These T cells regulate autoantibody production (Sinha et al, 1990). Thus, an imbalance in the regulation of the immune system causes the formation of autoantibodies against self antigens by B lymphocytes. However, low levels of non-pathological 'natural' autoantibodies may also be found in the normal state. Generally, susceptibility to autoimmunity is thought to be polygenic. The polymorphic amino acid residues in hypervariable allelic regions of the human leukocyte antigen (HLA) molecules have been shown to play an important role in determining susceptibility or resistance to autoimmune diseases. HLA molecules usually present peptide fragments of antigens to T cells. Different HLA molecules have different peptide-binding properties and are selective for particular T cells. Since T cells have both a diverse and a central role in the immune system, differences in HLA molecules may contribute to the development of an autoimmune disease. Accordingly, many such diseases have been associated with certain major histocompatibility alleles (Thorsby, 1997).

2.2.2. Pathophysiology of autoimmune thrombocytopenia

Platelet-specific autoantibodies may lead to enhanced Fc-mediated destruction of platelets by the mononuclear phagocytosing system, most commonly in the spleen, but also in the liver. The immunological nature of idiopathic thrombocytopenic purpura (ITP) was first confirmed by Harrington and co-workers (1951), who demonstrated a circulating 'anti-platelet factor' in human plasma as a possible cause of increased peripheral platelet destruction. Shulman et al (1965) confirmed these findings and reported that this anti-platelet factor was located in the IgG fraction.

Three major organs of the reticuloendothelial system, the spleen, the liver and the bone marrow, are critical in the pathological mechanisms of thrombocytopenia. The spleen is the most important site of both antibody production and platelet destruction, because of its optimal circulation and platelet sequestration. In patients with ITP, IgG production by the spleen has been shown to be increased (McMillan et al, 1974). Mizutani et al (1991) have reported a significant increase in circulating and splenic CD5+ B cells in these patients; the increase is correlated with the production of antiplatelet antibodies. However, splenectomised patients may show persistent thrombocytopenia. The liver, also, seems to play a pathological role, but probably only in severe forms of thrombocytopenia (Panzer et al, 1986; Najean et al, 1997).

Furthermore, platelet antibodies may have other effects. These antibodies may trigger platelet activation via platelet FcRs. For instance, GP IIbIIIa-specific monoclonal antibodies have been suggested to activate platelets via the platelet FcγRIIA (Anderson et al, 1991; Rubinstein et al, 1991; Berndt et al, 1993; Deckmyn et al, 1998). Platelet antibodies may also affect the megakaryocytes in the bone marrow. Since megakaryocytes carry the same antigens as mature platelets, platelet antibodies may interact with the megakaryocytes leading to impaired platelet production. Patients with ITP and circulating GP Ib-specific antibodies have been demonstrated to have megakaryocytes that are normal in number but smaller in size than those with GP IIbIIIa-specific antibodies, suggesting that GP Ib-specific antibodies may impair platelet production (Hasegawa et al, 1995). However, in the afore-mentioned study, GP-specific platelet-associated IgG (PA-IgG) was not measured in these patients, although enzyme-linked immunosorbent assay (ELISA) for PA-IgG was positive. Megakaryocyte-associated IgG has also been studied (Nagasawa et al, 1995). Some patients with ITP (an estimated 20%) may additionally have impaired megakaryocytic function and depressed platelet turnover (Ballem et al, 1987).

Other factors may also contribute to and be associated with the mechanisms of thrombocytopenia caused by increased peripheral platelet destruction. Platelets normally express only major histocompatibility complex class I molecules. Most studies on patients with ITP show no deviation in the frequency of HLA class I A, B or C antigens (McMillan et al, 1980; Gaiger et al, 1994). However, increased incidences of various HLA types have been reported to occur in these patients (Helmerhorst et al, 1982a; Gratama et al, 1984; Porges et al, 1985; Mueller-Eckhardt et al, 1989; Nomura et al, 1998). HLA-B8 and DR3, carrying DQA2.1 and DQB2.1, have been demonstrated to be overrepresented in patients with rheumatoid arthritis and gold-induced thrombocytopenia or proteinuria than in patients with rheumatoid

arthritis but without these side effects or in normal subjects (Singal et al, 1990). These inconsistencies are probably partly explained by a significant heterogeneity among patients with autoimmune thrombocytopenia.

There is also some evidence that genetic association of platelet alloantigens with autoimmune-mediated thrombocytopenia may exist. Song et al (1997) demonstrated an association of the genotype HPA-5a5b in GP Ia with autoimmune thrombocytopenia. Of the HPA-1, -2, -3 and -5 systems tested, HPA-2a allele was shown to be associated with chronic refractory ITP (Thude et al, 1999). However, the evidence of genetic association between autoimmune thrombocytopenia and the HPA system remains weak.

2.2.3. Different forms of thrombocytopenia

2.2.3.1. Idiopathic thrombocytopenia

Idiopathic thrombocytopenic purpura (ITP) is defined as isolated thrombocytopenia with no clinically apparent associated conditions or other causes of thrombocytopenia. Laboratory findings show isolated thrombocytopenia and the spleen size is normal. The incidence of ITP is estimated to be 10 to 125 new cases per 1 000 000 persons per year (George et al, 1996). Some patients may be asymptomatic, but usually the spontaneous bleeding manifestations, such as recurrent epistaxis, menorrhagia, mucosal bleeding, or skin ecchymoses, petechiae and purpura are associated with counts of between 10 and 50 x 10^9/l (Doan et al, 1960). Patients with platelet counts of less than 10 x 10^9/l are likely to have mucosal bleeding and a risk of central nervous system bleeding. The diagnosis of ITP is one of exclusion. Recently, The American Society of Hematology established a practice guideline for diagnostic criteria in ITP (George et al, 1996).

Among adults, ITP is typically a chronic disease lasting over 6 months, and spontaneous remission is infrequent. It is most frequent in young women, 70% of female patients being under 40 years of age (George et al, 1994). ITP in adults is a serious problem with a higher mortality rate than in children; the overall mortality rate associated with ITP is about 4 to 5% (Berchtold & McMillan, 1989b; Stasi et al, 1995; George et al, 1996). Patients with chronic refractory thrombocytopenia are known to be clinically heterogeneous. In adult patients, it is important to exclude other forms of immune-mediated thrombocytopenia and, for instance, myelodysplastic syndrome, aplastic anaemia, chronic lymphocytic

leukaemia and bone marrow infiltration as other causes of thrombocytopenia. The possibility of hereditary thrombocytopenias, such as Bernard-Soulier syndrome or Wiskott-Aldrich syndrome, should always be considered carefully, although hereditary thrombocytopenia may be difficult to distinguish from ITP.

2.2.3.2. Drug-induced thrombocytopenia

In drug-induced thrombocytopenia, the platelet count decreases after ingestion of the drug and usually starts to reverse shortly after cessation. The drugs most commonly associated with immune-mediated thrombocytopenia include quinine, quinidine, gold and heparin (Rizvi et al, 1999). Early appearance of thrombocytopenia may be seen, for instance, with sulphonamides, whereas a long induction period is typical of gold salts. Severe thrombocytopenia with acute onset and a high frequency of haemorrhage may be recorded during treatment with gold salts, sulphonamides, quinine and quinidine (Pedersen-Bjergaards et al, 1998). Different pathophysiological mechanisms for drug-induced thrombocytopenia have been proposed. A drug may conjugate with a platelet membrane protein and trigger production of hapten-dependent antibodies, or it may induce the expression of a neoantigen subsequently recognised by circulating antibodies only in the presence of the drug (Aster, 1999).

Gold-induced thrombocytopenia. Thrombocytopenia occurs in 1 to 3% of patients treated for rheumatoid arthritis with either parenteral or oral forms of gold salts (Mettier et al, 1948; Kosty et al, 1989). Usually the drug is taken over a period of 10 to 20 weeks before thrombocytopenia develops, but the onset may also be abrupt (Klimiuk et al, 1987). The duration of the thrombocytopenia varies, and recovery usually takes several weeks or months after cessation of the drug. In a few cases, the thrombocytopenia has persisted for years, which is suggested to be due to the long half-life of gold salts. Thrombocytopenia associated with gold therapy is suggested to be due to immune-mediated peripheral cell destruction mechanisms. Platelet survival is shortened, and the megakaryocyte count in the bone marrow has been demonstrated to be normal or increased (Levin et al, 1975; Klimiuk et al, 1987). Even IgG production by splenic leucocytes has been demonstrated to be enhanced (Levin et al, 1975; von dem Borne et al, 1986a). However, gold-induced thrombocytopenia may be difficult to differentiate from thrombocytopenia associated with the underlying disease.

Quinidine/quinine-induced thrombocytopenia. Immune-mediated thrombocytopenia is a well-recognised complication of quinidine and quinine therapy. The thrombocytopenia is usually severe and complicated by bleeding. Bleeding symptoms appear acutely during quinidine therapy, which may have continued for months or only for a few days. Quinidine-specific antibodies against platelets may be detectable in the presence of the drug (Bolton, 1956; Kekomäki et al, 1980).

Heparin-induced thrombocytopenia (HIT). The potentially life-threatening, immune-mediated HIT may be associated with both venous and arterial thrombotic events. It occurs in approximately 0.5 to 6.5% of patients treated with unfractionated heparin (Warkentin et al, 1997). Generally, the diagnostic criteria include both the clinical events and a positive laboratory test for heparin-dependent IgG. However, only a minority of patients with HIT antibodies develop clinical HIT. In HIT, a macromolecular complex composed of heparin (or low-molecular-weight-heparin or highly sulphated oligosaccharides) and platelet factor 4 has been demonstrated to serve as an antigen for the immunoglobulin (Greinacher et al, 1994a & b; Amiral et al, 1995). Interactions of the immunoglobulin with the platelet FcγRIIA, by cross-linking of FcγRIIs, will trigger intravascular platelet activation and lead to increased destruction of peripheral platelets (Anderson et al, 1991). Platelet FcR function (Warkentin et al, 1992) and density (Chong et al, 1993) may be correlated with the reactivity of platelets with HIT antibody. The distribution and association of the functional polymorphism of platelet FcγRIIA has also been studied in HIT patients, with inconsistent results.

2.2.3.3. Disease-related thrombocytopenia

Thrombocytopenia of autoimmune-mediated mechanisms has also been shown to be associated with various disorders, especially with other autoimmune diseases. In collagenosis, for example, immune thrombocytopenia is often present. In patients with systemic lupus erythematosus (SLE), mild thrombocytopenia is common, and severe symptomatic thrombocytopenia occurs in approximately 5%. Thrombocytopenia is even more common in patients with antiphospholipid antibodies (McNeil et al, 1991; Galli et al, 1994; Pujol et al, 1995).

Thrombocytopenia is often related to lymphoproliferative disorders. Patients with chronic lymphocytic leukaemia may develop thrombocytopenia due to splenic sequestration, secondary to marrow packing, but also due to immune destruction. Autoimmune-mediated thrombocytopenia has been estimated to

occur in 2 to 3% of patients with chronic lymphocytic leukaemia (Diehl & Ketchum, 1998). In lymphomas, thrombocytopenia may be related to marrow replacement, hypersplenism or an autoimmune mechanism; purpura has been shown to occur in up to two-thirds of patients with non-Hodgkin lymphoma or Hodgkin's disease.

Thrombocytopenia may also exist in association with autoimmune haemolytic anaemia, as in Evans' syndrome. In rheumatoid arthritis and inflammatory bowel disease, thrombocytopenia is mostly observed as a side effect of therapy, but these diseases are rarely reported in association with immune-mediated thrombocytopenia. The association of thyroid autoimmune disease and thrombocytopenia has also been recognised; it has been suggested that over 10% of patients with autoimmune-mediated thrombocytopenia might develop hyperthyroidism (Cordiano et al, 1998).

2.2.3.4. Thrombocytopenia in pregnancy

Thrombocytopenia may be observed in about 7% of pregnancies (McCrae et al, 1992). Mostly, the aetiology of thrombocytopenia in pregnancy is chronic ITP, incidental thrombocytopenia of pregnancy (gestational thrombocytopenia) or a complication of pregnancy. As a preliminary, preeclampsia or eclampsia, disseminated intravascular coagulation, thrombotic microangiopathy and other complications of pregnancy should always be excluded.

The most common cause of a low platelet count is incidental thrombocytopenia of pregnancy, accounting for 75% of cases of maternal thrombocytopenia (Burrows & Kelton, 1993). It is characterised by asymptomatic, mild (generally $>50 \times 10^9$/l) thrombocytopenia, with no previous history. It is usually present during late pregnancy, and resolves spontaneously postpartum, but may recur in subsequent pregnancies.

Previously, ITP was estimated to affect only 1 to 2 out of every 10 000 pregnancies, but the most recent reports suggest that the true incidence of ITP in pregnancy is higher, even 1 or 2 in every 1 000 pregnancies (McCrae et al, 1992; Burrows & Kelton, 1993; Sainio et al, 1998). ITP accounts for 3% of all cases of thrombocytopenia at the time of delivery (Burrows & Kelton, 1993). The diagnosis of ITP is especially difficult during pregnancy, since there are no particular features to distinguish it from the incidental thrombocytopenia of pregnancy.

2.2.3.5. Virus-induced thrombocytopenia

Viruses have been implicated in the aetiology of autoimmune diseases, and several mechanisms have been suggested to induce autoimmune responses (Kaplan et al, 1992). The major mechanisms involved in virus-induced immune thrombocytopenia are suggested to be disturbance in the host immune response, molecular mimicry, production of anti-idiotype antibodies, enhanced expression of the major histocompatibility complex molecules and changes in endogenous antigen. Platelet injury may also result from antigen-antibody complexes originating from an immunological event essentially unrelated to the platelet, i.e. the 'innocent bystander' hypothesis.

In asymptomatic patients infected with the human immunodeficiency virus, the incidence of thrombocytopenia may be 0 to 10%, whereas in symptomatic patients thrombocytopenia may be found in up to 40% (Kaplan et al, 1992). Thrombocytopenia is a frequent manifestation in patients with rubella (1/3000), whereas it seems to occur less frequently in measles and mumps (Nieminen, 1992). Mild to moderate thrombocytopenia may be present in half the patients with Epstein-Barr virus infection. Thrombocytopenia is also sporadically found in adult patients infected with cytomegalovirus. Acute thrombocytopenia may also develop shortly after morbilli-mumps-rubella vaccination (Nieminen, 1992).

2.2.3.6. Cyclic thrombocytopenia

Cyclic thrombocytopenia is a rare disorder characterised by the alternation at regular intervals of thrombocytopenia and thrombocytosis. It may be due to a variety of mechanisms, but a form with an immunological mechanism has been suggested to exist. Patients are usually women whose oscillations may correlate with the menstrual cycle (Tomer et al, 1989). Autoimmune antibodies have also been reported to be involved in the aetiology of this disorder (Menitove et al, 1989).

3. DETECTION OF PLATELET-ASSOCIATED ANTIBODIES

3.1. Flow cytometry as a diagnostic device

Flow cytometry allows rapid analytic measurement of large quantities of particles. A suspension of fluorescence-labelled cells is injected into a flowing stream of fluid that passes through a focused laser beam. Each cell transversing the laser beam will scatter light and generate emitted signals that are collected through appropriately arranged filters and photodetectors. The light scatter signals, forward light scatter (FSC) and side light scatter, provide information about cell size and cytoplasmic and nuclear characteristics, respectively.

Different fluorescent signals may be collected in separate detection channels simultaneously. The fluorescent dye used should absorb strongly at the wavelengths for which the excitation source is available. The fluorescent labels most commonly used are fluorescein isothiocyanate, which gives green fluorescence, and phycoerythin, which gives red-orange fluorescence. For example, in the detection of platelet antibodies or platelet glycoproteins, the fluorescent dye may be coupled with an antibody. A fluorescent dye, thiazole orange (TO), which gives green fluorescence, binds to both DNA and RNA and forms a fluorescent nucleotide-reagent complex (Lee et al, 1986). It may be used to detect platelets with an increased nucleic acid content, reticulated platelets (RP). For diagnostic purposes, flow cytometry offers numerous possibilities.

3.2. Platelet-associated IgG (PA-IgG)

3.2.1. Platelet antibodies

In autoimmune thrombocytopenia, it is agreed that patients' autologous platelets rather than circulating antibodies should be studied when detecting platelet autoantibodies (Brighton et al, 1996; Kiefel et al, 1996; Berchtold et al, 1997; Warner et al, 1999). Normally, platelets are known to contain approximately 5 fg of IgG per platelet, of which only 1% is found on the platelet surface, and the majority of the total platelet IgG is within secretory α-granules (George, 1991; LoBuglio et al, 1983; Leissinger & Stuckey, 1992). The increase in PA-IgG has been shown to be closely related to the reduced platelet count. However, occasionally platelet autoantibodies may be present even if the platelet count is normal. PA-IgG has been demonstrated to be inversely proportional to the platelet count and to the mean platelet lifespan (Dixon et al, 1975; Mueller-Eckhardt et al, 1980; Panzer et al, 1986; Nieminen, 1992; Movahed

Shariat Panahi et al, 1994). PA-IgG is known to consist mostly of subclasses IgG1 and IgG3 (von dem Borne et al, 1980; Taaning & Petersen, 1988; Tijhuis et al, 1991).

Although in the diagnostics of autoimmune thrombocytopenia the detection of circulating platelet antibodies is no longer considered sensitive or feasible, the knowledge previously gained, however, is mostly about circulating antibodies. Detection of antibodies has revealed that GP IIbIIIa and GP IbIX are the most immunogenic of the platelet glycoproteins (van Leeuwen et al, 1982; Beardsley et al, 1984; Woods et al, 1984a & b; McMillan et al, 1987; Tomiyama et al, 1987 & 1989; Berchtold et al, 1989a; Kokawa et al, 1993; Deckmyn et al, 1994; He et al, 1994; Stockelberg et al, 1995; Kiefel et al, 1996). Furthermore, circulating platelet antibodies directed against GP IaIIa have been demonstrated occasionally (Deckmyn et al, 1990; Godeau et al, 1997). Only a few data have been published on GP V as a target for circulating platelet antibodies. Stricker & Shuman (1986) reported six patients with quinidine-induced thrombocytopenia who had GP V-specific quinidine-dependent platelet antibodies. The platelet antibodies were detected in serum by an immunoblotting assay with purified GP V as a target. Bernard-Soulier platelets did not react with the antibody, the binding of which was Fab-mediated; all this indicated that the target antigen was GP V. GP V-specific IgM antibodies characterised and detected by an immunoblot analysis were reported in three children with clinically diagnosed varicella zoster virus infection (Mayer & Beardsley, 1996).

In addition, a number of circulating antibodies directed against different platelet GPs have been identified, for instance, in patients with human immunodeficiency virus and Epstein-Barr virus infections (Winiarski, 1989; Gonzalez-Conejero et al, 1996). In patients with rubella, varicella zoster virus, human immunodeficiency virus and Epstein-Barr virus infections, circulating autoantibodies and also PA-IgG have been detected (Winiarski, 1989; Bettaieb et al, 1992; Kaplan et al, 1992; Gonzalez-Conejero et al, 1996).

Drug-dependent antibodies in sera of patients with quinidine- or quinine-induced thrombocytopenia have mostly been reported to react with GP IIbIIIa or GP IbIX (Kunicki et al, 1978; van Leeuwen et al, 1982; Christie et al, 1987; Visentin et al, 1991; Burgess et al, 1998). GP IbIX-specific drug-dependent antibodies were associated with an acute, severe and quickly reversing type of thrombocytopenia, and GP IIbIIIa-specific antibodies were associated with positivity for PA-IgG (Nieminen & Kekomäki, 1992). In a few cases, gold-dependent antibodies have been demonstrated to be present (Kosty et al, 1989;

Adachi et al, 1987), but they have mostly been undetectable (Stavem et al, 1968; Levin et al, 1975; von dem Borne et al, 1986a; Klimiuk et al, 1987).

Platelet antibody targets have also been localised on unidentified proteins, glycolipids, glycosphingolipids and phospholipids (van Vliet et al, 1987; Jouhikainen et al, 1990). A meta-analysis showed that 30% of ITP patients have antiphospholipid antibodies (McNeil et al, 1991). Reid et al (1990) demonstrated that the majority of normal sera contain IgG binding to protein bands at 90 to 95 kDa. Circulating antibodies against internal platelet proteins, such as vinculin and talin (Tomiyama et al, 1992; Reid et al, 1993), and also against cytoskeletal tropomyosin (Hou et al, 1996) have been demonstrated in both patients and controls. Platelet granule membrane protein of 140 kDa, GMP140 (CD62) has also been reported to be a target (Bierling et al, 1994). The pathogenetic significance of antibodies against relatively inaccessible targets remains to be determined. Thus, the importance of circulating antibodies should be interpreted with caution.

3.2.2. Detection of PA-IgG

Direct assays measure platelet-bound immunoglobulins, antibodies on the surface of intact platelets. For the first time, the quantitative antiglobulin consumption assay allowed the direct determination of PA-IgG (Dixon et al, 1975). In this test, platelet-bound IgG was determined from the consumption of anti-IgG, as measured by inhibition of the lysis of IgG-coated sheep red cells in the presence of complement. However, in the detection of PA-IgG, the platelet immunofluorescence test (PIFT) and the ELISA have been applied widely (von dem Borne et al, 1978; Lin et al, 1990; Table 3). In PIFT, a paraformaldehyde-fixed platelet suspension is incubated with fluorescence-labelled anti-human antibody and analysis of the fluorescence may be performed with flow cytometry. There is a wide variety of different ELISA methods. In general, a platelet suspension is attached to microtitre wells as a monolayer or as a detergent lysate, and antibodies, tagged with peroxidase or alkaline phosphatase serve as ligands. Moreover, in the platelet radioactive antiglobulin test, radioactive ^{125}I-labelled anti-IgG is bound to the IgG on the platelet surface, and the amount of radioactivity is measured to obtain the amount of PA-IgG (Soulier et al, 1975; Mueller-Eckhardt et al, 1980; Table 3).

Table 3. Studies on platelet-associated IgG (PA-IgG)

Method	Diagnosis	Patients n	PA-IgG n (%)	Reference
PIFT	ITP	80	55 (69)	von dem Borne et al, 1980
PRAT	TP	176	94 (53)	Mueller-Eckhardt et al, 1980
QACA	Drug-induced TP	18	18 (100)	Kelton et al, 1981
PIFT	Immune TP Disease-related TP	215 142	195 (91) 136 (96)	Helmerhorst et al, 1982b
PIFT (direct eluate)	RA with gold therapy	13	13 13 eluate	von dem Borne et al, 1986a
PIFT	ITP TP	75 194	63 (84) 153 (79)	von dem Borne et al, 1986b
PRAT	ITP	17	11 (65)	Panzer et al, 1986
FCM	ITP	44	33 (75)	Rosenfeld et al, 1987a
PRAT	Immune TP Non-immune TP	67 55	60 (90) 4 (7)	Court et al, 1987
ELISA	TP (children)	17	16 (94)	Taaning & Petersen, 1988
PIFT	ITP	109	88 (81)	Nieminen, 1990
Immunobead assay	ITP children Non-TP children	36 31	26 (72) 15 (48)	Imbach et al, 1991
PIFT	Quinidine-induced TP	14	7 (50)	Nieminen & Kekomäki, 1992
FCM	Immune TP Non-immune TP	16 9	12 (75) 2	Christopoulos et al, 1993
FCM	SLE ITP	25 65	11 (44) 45 (69)	Kurata et al, 1993
ELISA	ITP	33	28 (85)	Movahed Shariat Panahi et al, 1994
ELISA	ITP	30	11 (37)	Nagasawa et al, 1995
PRAT	Chronic liver disease	33	17 (52)	Pereira et al, 1995
CELIA	Immune TP Non-immune TP	81 46	60 (74) 34 (74)	Brighton et al, 1996
FCM, ELISA	HIV	45	19 (42) FCM 16 (36) ELISA	Gonzalez-Conejero et al, 1996
PRAT	MDS	30	21 (70)	Hebbar et al, 1996
Immunoradio-metric assay	ITP Non-immune TP	49 32	38 (78) 26 (81)	Warner et al, 1999

CELIA: competitive ELISA; ELISA: enzyme-linked immunosorbent assay; FCM: flow cytometry, immuno-fluorescence assay; HIV: human immunodeficiency virus; ITP: idiopathic thrombocytopenic purpura; MDS: myelodysplastic syndrome; PIFT: platelet immunfluroescence test; PRAT: platelet radiolabelled antiglobulin test; QACA: quantitative antiglobulin consumption assay; RA: rheumatoid arthritis; TP: thrombocytopenia

The direct PIFT has been considered especially sensitive; 70 to 85% of patients with ITP have been demonstrated to have positive PA-IgG with direct PIFT (Table 3). Christopoulos et al (1993) used a similar assay based on flow cytometry, and demonstrated positive values of PA-IgG in 75% (12/16) of patients suggested to have immune thrombocytopenia and in 2 of 9 patients with suspected non-immune thrombocytopenia (Table 3). Helmerhorst et al (1980) compared the three assays, PIFT, the quantitative antiglobulin consumption assay and the platelet radioactive antiglobulin test, for the detection of PA-IgG. They found PIFT to be the most sensitive technique. Yet, in the diagnosis of autoimmune thrombocytopenia detection of PA-IgG suffers from lack of specificity (von dem Borne et al, 1986b; Rosenfeld et al, 1987a). However, complications in the measurement of platelet antibodies may be caused by naturally occurring antibodies, internal platelet IgG and aggregated IgG of immune complexes bound to the platelet or antibodies against platelet cryptantigens (Rosenfeld et al, 1985; Muniz-Diaz et al, 1995).

3.2.3. PA-IgG in patients of different groups

PA-IgG has been detected on the platelet membrane in 75 to 90% of patients with ITP (Dixon et al, 1975; LoBuglio et al, 1983; McMillan et al, 1987; Tani et al, 1989; Berchtold & Wenger 1993). Some other studies on PA-IgG are listed in Table 3. However, the specificity of PA-IgG in the diagnosis of ITP is less than 30% (Mueller-Eckhardt et al, 1980; Kelton et al, 1982).

Furthermore, PA-IgG is often present in gold-induced thrombocytopenia (Kelton et al, 1981; von dem Borne et al, 1986a; Adachi et al, 1987; Kosty et al, 1989), although its GP specificity has not been determined. Moreover, increased PA-IgG has been reported in varying proportions of patients with thrombocytopenia that is presumably not of immunological origin (Helmerhorst et al, 1982b; Kelton et al, 1982). With the quantitative platelet radioactive antiglobulin test, Mueller-Eckhardt et al (1980) demonstrated large amounts of PA-IgG in patients with systemic blood diseases, such as leukaemias or lymphomas and hepatic or infectious disorders. Warner et al (1999) reported PA-IgG by immunoradiometric assay in 78% (of 49) patients with ITP, but also in 81% (of 32) patients with non-immune thrombocytopenia, such as pancytopenia secondary to chemotherapy, aplastic anaemia, Bernard-Soulier syndrome, incidental thrombocytopenia of pregnancy, platelet function defect, human immunodeficiency virus and hepatitis. Occasionally in the case of hereditary thrombocytopenia, PA-IgG may be detectable, probably because of the platelet membrane abnormalities.

Platelet autoantibodies of the IgM and IgA classes have been suggested to play only a minor role in the pathogenesis of thrombocytopenia caused by increased platelet destruction. Yet, autoantibodies of all three Ig classes have been reported to correlate with the platelet count (Kokawa et al, 1991; Movahed Shariat Panahi et al, 1994). With the PIFT PA-IgG, PA-IgM and PA-IgA were detected in 92%, 42% and 9% of patients with ITP, respectively (von dem Borne et al, 1986b). Kiefel et al (1996) detected GP-specific PA-IgG, PA-IgM and PA-IgA in 46%, 15% and 3% of patients with autoimmune-mediated thrombocytopenia. In a few cases, PA-IgM and PA-IgA may offer additional information, but their presence is not yet fully understood.

3.3. GP-specific PA-IgG

3.3.1. Detection of GP-specific PA-IgG

Different applications of monoclonal antibodies specific for specific platelet proteins have made it possible to define the platelet antibody targets clearly. Generally in antigen capture assays, platelet antigens are solubilised and immobilised on plastic wells prior to exposure to the antibody. Woods et al (1984a) used a microtitre well assay, in which platelet GPs were fixed to microtitre plates with monoclonal antibodies. The wells were exposed to the patient's serum and binding of the antibody directed against GP was determined with ^{125}I-labelled anti-human IgG. Later on, monoclonal antibody-specific immobilisation of platelet antigens (MAIPA) allowed detection of the GP specificity of platelet antibodies (Kiefel et al, 1987).

The GP specificity of PA-IgG may be detected with different direct methods, such as MAIPA, immunobead assay, immunoprecipitation and modified antigen capture ELISA (MACE; McMillan et al, 1987; Tomiyama et al, 1990; Kiefel et al, 1991; Berchtold & Wenger, 1993; Berchtold et al, 1997; Fig 4). In the antigen capture assay, platelet-specific monoclonal antibodies capture an epitope carrying molecules from a platelet lysate onto a solid matrix, and their ligands are labelled. The direct modified antigen capture assay, based on this principle, is similar to MAIPA and the immunobead assay methods (Kurata et al, 1993; Pereira et al, 1995; Berchtold et al, 1997). The MAIPA and the immunobead assay are the tests most widely used for the measurement of GP-specific PA-IgG (Table 4).

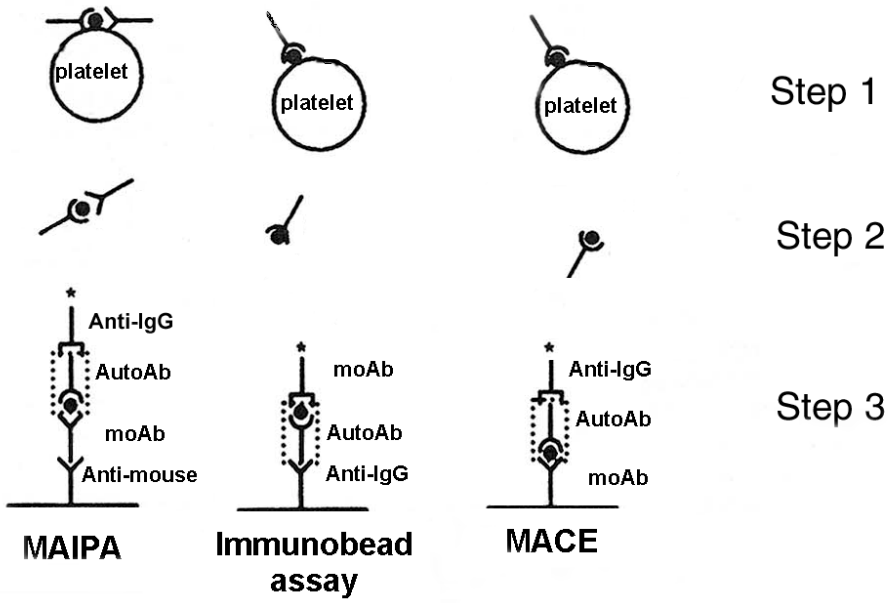

Fig 4. Platelet glycoprotein (GP)-specific assays for the direct detection of GP-specific platelet-associated IgG (PA-IgG). Washed platelets with GP-specific PA-IgG (step 1) are lysed (step 2) and the GP of interest is immobilised on a plastic surface using a monoclonal antibody (moAb) or an Ab specific against human IgG, (Anti-IgG; step 3). In contrary, in MAIPA, the sensitisation with moAb is performed prior the cell lysis. Anti-mouse: Ab against mouse IgG; AutoAb: platelet-specific autoAb; MACE: modified antigen capture ELISA; MAIPA: monoclonal antibody-specific immobilization of platelet antigens.

In the direct immunobead assay, the patient's washed platelets are solubilised and the centrifuged supernatant containing the immune complexes is captured on a plastic surface using a murine platelet GP-specific monoclonal antibody (Fig 4; McMillan et al, 1987; Berchtold et al, 1989b; Tani et al, 1989; Berchtold et al, 1997). Either polystyrene beads or microtitre wells may be used as a plastic surface. In contrast to the MAIPA, in which the patient's platelets are first incubated with a monoclonal antibody, solubilisation is performed prior to incubation.

In the direct MAIPA (Kiefel et al, 1991 & 1996), washed platelets are incubated with monoclonal antibody. After solubilisation, incubation is performed on a plastic surface coated with anti-mouse Ig, and the captured complex is detected with enzyme-labelled anti-human IgG (Fig 4). Different forms of the MAIPA assay have already been introduced (Kiefel, 1992). The MAIPA assay may be considered both sensitive and reliable, although it has certain limitations, such as false-negative results due to

epitope competition between human antibodies and monoclonal antibodies (Morel-Kopp et al, 1996). On the one hand, selection of the monoclonal antibodies used and, on the other hand, lack of specific monoclonal antibodies are also important factors restricting the value of MAIPA. Brighton et al (1996) used the direct MAIPA for the detection of GP IIbIIIa- and GP IbIX-specific PA-IgG in randomly chosen patients with thrombocytopenia initially diagnosed to be either immune (n=94; ITP, disease-related and drug-induced thrombocytopenia) or non-immune (n=53; aplastic anaemia, neoplastic infiltration, chronic liver disease, drug-associated thrombocytopenia). As reference methods for PA-IgG, they used both ELISA and a modified competitive ELISA, and they observed a sensitivity of 51% and a specificity of 80% with MAIPA.

Both the direct MAIPA and the direct immunobead assay are sensitive, reproducible and consistent in their results. The first international study to compare methods for the detection of GP-specific PA-IgG was reported in 1997 (Berchtold et al). Blinded samples, supplied by participants, were centrally aliquoted and distributed. Eight participating laboratories used either MAIPA, the immunobead assay or MACE to detect GP-specific PA-IgG. It was concluded that good agreement was obtained between the laboratories in the detection of GP-specific PA-IgG. Crossley et al (1997) compared the MAIPA and immunobead assays for the detection of GP IIbIIIa-specific PA-IgG. The study showed MAIPA assay to be more reproducible than the immunobead assay (9/10 vs. 6/10 identical results repeatedly parallel, respectively), and the sensitivities (although tested with titrated positive serum) were similar. Recently, MACE and MAIPA were compared for the detection of GP IIbIIIa-specific PA-IgG of 81 samples from thrombocytopenic patients (Warner et al, 1999). The GP-specific assays were found to be alike in sensitivity (39%) and specificity (91%). In agreement, direct GP-specific assays may complement PA-IgG measurements in the classification of autoimmune-mediated thrombocytopenia.

Direct platelet eluates also allow detection of GP-specific PA-IgG and the results obtained are in good agreement with direct tests; in 82 ITP patients, the antibodies were shown to have specificities against both GP IIbIIIa (in up to 90%) and IbIX (less than 20%; Kiefel et al, 1996; Hürlimann-Forster et al, 1997; Table 4). Nevertheless, the detection of direct platelet eluates requires a selected patient population with a relatively high platelet count.

Table 4. Studies on glycoprotein (GP)-specific platelet-associated IgG (PA-IgG)

Method	Diagnosis	Patients n	GP-specific PA-IgG n (%) IIbIIIa	IbIX	IIbIIIa/IbIX	Reference
Immunobead assay	ITP	28	13 (46)	8 (29)	21 (75)	McMillan et al, 1987
Immunobead assay	Disease-related TP	19	9 (47)	2 (11)		Berchtold et al, 1989c
Immunoprecipitation	ITP	6	4			Tomiyama et al, 1990
Immunobead assay	ITP children ITP adults	41 16	40 (98) 14 (88)	1 (2) 2 (13)		Imbach et al, 1991
MAIPA	Immune TP	29	21 (72)	22 (28)	14 (48)	Kiefel et al, 1991
ELISA	ITP	71	25 (35)	10 (14)		Kokawa et al, 1991
Immunobead assay	ITP In remission	53 17	26 (49) 10 (59)	6 (11) 3 (18)	7 (13) 4/17 (24)	Berchtold & Wenger, 1993
MACE (direct eluate)	ITP	7	6			Kurata et al, 1993
MACE	Chronic liver disease	36	3 (8)	10 (28)	6 (17) 19 (58)	Pereira et al, 1995
MAIPA	Immune TP Non-immune TP	81 51	19 (23)	5 (6)	16 (20) 11 (22)	Brighton et al, 1996
MAIPA	Immune TP Non-immune TP	78 40			36 (46) 0	Kiefel et al, 1996
PAICA	ITP	20	8 (40)	2 (10)	2 (10)	Macchi et al, 1996
Immunobead assay, MAIPA, MACE	TP	22	18 (82)	17 (77)		Berchtold et al, 1997*
Immunobead assay, MAIPA	ITP	40	8 (20)			Crossley et al, 1997
ELISA (direct eluate)	ITP	82	75 (92)	14 (17)		Hürlimann-Forster et al, 1997
MAIPA, Antigen capture assay	ITP Non-immune TP	49 32	19 (39) 3 (9)		19 (39) 3 (9)	Warner et al, 1999

* A total of eight participating laboratories, taken as positive when reported positive by >2 laboratories.
ELISA: enzyme-linked immunosorbent assay; ITP: idiopathic thrombocytopenic purpura; MACE: modified antigen capture ELISA; MAIPA: monoclonal antibody-specific immobilization of platelet antigens; PAICA: platelet-associated IgG characterization assay; PIFT: platelet immunofluorescence test; TP: thrombocytopenia.

3.3.2. Antigens and epitopes of PA-IgG

Studies on GP IIbIIIa- and IbIX-specific PA-IgG are summarised in Table 4. As specific autoepitopes, PA-IgG has also been determined to be directed against cation-dependent conformational antigens (Fujisawa et al, 1993). Using recombinant peptides, one patient with GP IIbIIIa-specific PA-IgG was demonstrated to have antibodies binding to the region of amino acids 350-550 of GP IIIa. Kosugi et al (1996b) were able to recognise cation-dependent regions of GP IIbIIIa as autoantigenic targets of PA-IgG. The presence of GP IV-specific PA-IgG was detected with the immunobead assay in one patient with a bleeding disorder who also had both GP IIbIIIa- and GP IaIIa-specific autoantibodies (Beer et al, 1993).

3.3.3. GP-specific PA-IgG in patients of different groups

Several studies have shown that direct measurements of GP-specific platelet antibodies may be used in the classification of autoimmune thrombocytopenia (Table 4). McMillan et al (1987) demonstrated PA-IgG in 75% of patients with ITP, 62% of whom had GP IIbIIIa and 58% GP Ib specificity. Similarly, Tani et al (1989) found GP IIbIIIa- and/or GP IbIX-specific PA-IgG in 80% of patients with ITP. Detection of the glycoprotein (GP) specificity of platelet-associated IgG (PA-IgG) has been suggested to improve the specificity of tests aimed at the detection and classification of autoimmune thrombocytopenia.

GP-specific PA-IgG has been detected not only in patients with ITP, but also in patients with disease-related thrombocytopenia, i.e. SLE, chronic lymphocytic leukaemia or chronic liver disease. Berchtold et al (1989c) were able to detect GP IIbIIIa- and GP IbIX-specific PA-IgG in 60% of patients with disease-related thrombocytopenia, such as chronic lymphocytic leukaemia, Hodgkin’s disease, idiopathic pulmonary fibrosis, mixed connective tissue disease and Sjögren's syndrome.

In patients with SLE, the PA-IgG of platelet eluates has been shown to bind specifically to GP IIbIIIa, and the presence of PA-IgG was significantly associated with thrombocytopenia and disease activity (Kurata et al, 1993). Fabris et al (1994) demonstrated PA-IgG by direct PIFT in 6 of 10 patients with SLE and in 5 of 8 patients with primary anti-phospholipid syndrome. In another study of 90 patients with SLE, the prevalence of PA-IgG was also high (62%; Pujol et al, 1995).

A high prevalence of GP-specific PA-IgG has also been observed with MACE in patients with chronic liver disease (Pereira et al, 1995; Table 4). Using a direct platelet eluate, GP IIbIIIa-specific antibodies were demonstrated in a patient with primary biliary cirrhosis (Panzer et al, 1990). Also using direct platelet eluates, Bettaieb et al (1992) demonstrated GP IIbIIIa-specific PA-IgG in patients with human immunodeficiency virus. Direct MAIPA was positive in 5 of 8 patients with ITP, as well as in 4 of 8 patients with disease-related thrombocytopenia, such as SLE, and also in 2 of 4 patients with systemic autoimmune disease without thrombocytopenia (Cordiano et al, 1996).

Brighton et al (1996) reported GP IIbIIIa- and IbIX-specific PA-IgG by MAIPA in 49% (40/81) of patients with immune thrombocytopenia (ITP or secondary immune) and in 22% (11/51) of patients with an initial diagnosis of non-immune thrombocytopenia, including diagnoses such as non-Hodgkin's lymphoma, chronic liver disease, aplastic anaemia, leukaemia, multiple myeloma and disseminated intravascular coagulation. Warner et al (1999) reported GP IIbIIIa-specific PA-IgG detected by MACE and MAIPA in 39% (of 49) patients with ITP and also in three (chronic hepatitis and two with the myelodysplastic syndrome) of 32 patients with non-immune thrombocytopenia. Thus, detection of GP-specific PA-IgG seems to improve our understanding of the nature of platelet antibodies in the different forms of autoimmune thrombocytopenia and in forms of thrombocytopenia initially considered non-immune.

4. PLATELET Fcγ RECEPTOR TYPE IIA (FcγRIIA)

FcγRIIA is the only FcγR expressed on platelets (Rosenfeld et al, 1985; Cassel et al, 1993; Wu et al, 1996). The number of FcγRIIA molecules on the platelet surface may vary considerably between individuals; the number of binding sites per platelet has been suggested to vary between 600 and 8000 (Karas et al, 1982; Rosenfeld et al, 1987b; Anderson et al, 1995; Table 1). The average number of receptors per platelet has been shown to be stable. For complexed IgG, the binding affinity of platelet FcγR has been shown to be low (Kekomäki & Myllylä, 1979). Platelet FcγRIIA has been demonstrated to be topographically and probably also functionally associated with GP IIbIIIa, and GP IIbIIIa-specific monoclonal antibodies are suggested to activate platelets via FcγRIIA (Berndt et al, 1993; Rubinstein et al, 1991).

The only known functionally important genetic polymorphism of FcγRIIA is the polymorphism H131R, which has been shown in a three-dimensional computer-based model to be located near the potential IgG-binding site (Hulett et al, 1994). The genetic polymorphism of FcγRIIA in amino acid position 131 results from a single base substitution (A/G) at nucleotide 494 in the second extracellular domain of the receptor (Clark et al, 1989; Warmerdam et al, 1990). In the early studies, different affinity patterns of the structural polymorphism were demonstrated in monocytes (Anderson et al, 1987). The monoclonal antibody 41H16, directed against FcγRII, binds strongly only to the arginine 131 (R131) form of the FcγRIIA protein and thus discriminates between these two polymorphic forms. The histidine 131 (H131) allele (494A) is characterised by high affinities for human IgG2 and IgG3, whereas the R131 allele (494G) has a low affinity for human IgG2 (Warmerdam et al, 1991; Parren et al, 1992). IgG1 binds equally well to platelets expressing any of the FcγRIIA phenotypes (Warmerdam et al, 1991; Osborne et al, 1994).

4.1. Typing of FcγRIIA polymorphism H131R

Several methods have been introduced for the detection of FcγRIIA polymorphism. Functional assays were applied first to determine the different forms of FcγRIIA (Rosenfeld et al, 1987b). Phenotyping of FcγRIIA is possible with monoclonal antibodies (Salmon et al, 1992; Sanders et al, 1994; Duits et al, 1995). At present, detection of FcγRIIA polymorphism is most commonly performed by genotyping. Clark et al (1991) were the first to use selective oligonucleotide primers to amplify a DNA fragment encoding the FcγRIIA polymorphism site and perform DNA sequencing. Both automated DNA sequence analysis and a single-strand conformation polymorphism assay have been used after polymerase chain reaction (PCR) amplification of a 278-base pair (bp) FcγRIIA-specific fragment (Reilly et al, 1994). The method used after FcγRIIA-specific PCR amplification, allele-specific oligonucleotide hybridisation is probably the most widely used, since it was first introduced as an easily adaptable genotyping method (Osborne et al, 1994; Brandt et al, 1995; Burgess et al, 1995; Salmon et al, 1996; Williams et al, 1998). Bachelot et al (1995) used denaturing gradient gel electrophoresis and DNA sequencing of single-strand templates. De Haas et al (1995) used the 1000-bp fragment product of the initial FcγRIIA-specific PCR amplification (Osborne et al, 1994) in a nested PCR method, in which a sense mismatch primer created a novel potential restriction site.

A more rapid genotyping method, allele-specific restriction enzyme digestion, requires only one step PCR to introduce a potential restriction site (Jiang et al, 1996; Arepally et al, 1997; Atsumi et al, 1998; Lehrnbecher et al, 1999). A recently developed rapid genotyping method, using allele-specific primers, seems to give reliable results when compared with allele-specific oligonucleotide hybridisation (Flesch et al, 1998). Thus, several methods have been applied for FcγRIIA genotyping, the restriction enzyme digestion methods being the most reliable and easily adapted methods. Recently developed rapid methods will have an advantage over the earlier ones.

4.2. The clinical significance of platelet FcγRIIA polymorphism H131R

The HH131 genotype of FcγRIIA has been associated with enhanced clearance of immune complexes compared with HR131 and RR131; both polymorphonuclear cells and monocytes from HH131 donors bound and internalised human IgG2-coated erythrocytes efficiently, whereas phagocytes from RR131 donors did so poorly (Salmon et al, 1992). Although the H131R polymorphism has been shown to affect the ligand binding properties of the receptor for the different IgG subclasses, whether the H131R polymorphism affects the level of PA-IgG on circulating platelets from thrombocytopenic patients has not been studied. Since FcγRs have been suggested to play a role in causing susceptibility to or modification of some diseases, distribution of FcγRIIA polymorphism has also been studied widely in different groups of patients with SLE, HIT, myasthenia gravis, chronic granulomatous disease, sickle cell anaemia or bacterial infections (Lehrnbecher et al, 1999; Table 5). It seems that the R131 allele may have a potential role related to the nephritis in SLE (Duits et al, 1995; Salmon et al, 1996). The role of FcγRIIA polymorphism in HIT continues to be inconsistent (Table 5). In 29 patients with refractory ITP the distributions of FcγRIIA polymorphism showed skewness towards the RR131 genotype (Table 5). Thus, the clinical significance of the FcγRIIA polymorphism in the different immune-mediated thrombocytopenic conditions needs further evaluation.

Table 5. Distribution of genetic polymorphism H131R of FcγRIIA in patients with different diagnoses.

Diagnosis (ethnic origin)	Controls			Patients						Reference
	n	H131	R131	n	H131	R131	HH131 (%)	HR131	RR131	
SLE (Caucasian)	259	0.45	0.55	215	0.44	0.56	21	45	33	Botto et al, 1996
SLE	100	0.52	0.48	214	0.40	0.60	17	**45***	**37***	Salmon et al, 1996
SLE (Caucasian)	187	0.51	0.49	108	0.54	0.46	37	34	29	Manger et al, 1998
SLE nephritis	100	0.52	0.48	103	0.35	0.65	12	47	**42***	Salmon et al, 1996
SLE	69	0.58	0.42	95	0.45	0.55	19	53	28	Duits et al, 1995
SLE (British)	66	0.47	0.53	81	0.43	0.57	12	60	27	Smyth et al, 1997
SLE (Afro-Caribbean)	77	0.45	0.55	70	0.38	0.62	11	53	36	Botto et al, 1996
SLE nephritis	69	0.58	0.42	50	0.38	0.62	14	**48**	**38***	Duits et al, 1995
SLE (Chinese)	49	0.69	0.31	46	0.63	0.36	39	50	11	Botto et al, 1996
SLE (Greek)	52	0.62	0.38	42	0.52	0.48	33	38	29	Smyth et al, 1997
HIT	256	0.54	0.46	389	0.46	0.54	20	53	**27***	Carlsson et al, 1998
HIT	100	0.44	0.56	96	0.56	0.44	**34***	43	23	Brandt et al, 1995
HIT	264	0.47	0.53	84	**0.57***	0.44	36	41	23	Denomme et al, 1997
HIT	102	0.47	0.53	36	0.49	0.51	22	53	25	Arepally et al, 1997
HIT	218	0.56	0.44	25	0.52	0.48	20	64	16	Bachelot-Loza et al, 1998
HIT (Caucasian)	22	0.39	0.61	19	0.61	0.39	**21***	**79***	0	Burgess et al, 1995
Refractory ITP	61	0.53	0.47	29	0.31	0.69	10	42	**48***	Williams et al, 1998
Apl syndrome	41	0.50	0.50	100	0.46	0.54	21	50	29	Atsumi et al, 1998

* significantly overrepresented according to the original reference, as compared with the controls.
Apl: antiphospholipid; HIT: heparin-induced thrombocytopenia; ITP: idiopathic thrombocytopenic purpura; SLE: systemic lupus erythematosus.

5. DETECTION OF RETICULATED PLATELETS (RP)

Platelets with an increased nucleic acid content are believed to be young platelets, RP. In the circulation RP are suggested to reflect megakaryocyte stimulation and platelet production. RP can be detected with thiazole orange (TO; Lee et al, 1986) staining combined with flow cytometric analysis of the fluorescence (FL; Kienast & Schmitz, 1990). The TO labelling has been shown to be sensitive to ribonuclease (RNAase), and thus may be considered to be due to an increased RNA content (Ault et al, 1992).

5.1. Measurement of RP

One of the problems in direct measurement of TO-stained platelets is to define a threshold gate for positivity. Some studies on RP are summarised in Table 6. Ault et al (1992) defined the proportion of RP by drawing an arbitrary line using the FSC versus FL plot with the same slope as the platelet cluster to segregate 99% of the normal platelets below the line. The positioning of the line was shown to be consistent after RNAase treatment. An automated measurement of RP was based on this same principle (Watanabe et al, 1995). Bonan et al (1993) used autologous erythrocyte TO fluorescence in whole blood as an internal standard for TO fluorescence of platelets. Rinder et al (1993) used a threshold defined by normal control values, i.e. the mean for a normal control sample + 2 standard deviations (SD), as a limit for TO-positive platelets. Unstained control samples have also been used in setting the gate of positivity (Romp et al, 1994; Richards & Baglin, 1995; Tàssies et al, 1995). Recently, lyophilised platelets were also introduced as control samples (Saxon et al, 1998). However, there is no internationally agreed system of standardisation or consensus for acquisition of data or for their analysis.

5.2. RP in healthy controls and patients

In normal adults, the range of RP has been reported to vary greatly between 1 and 20% including inter-individual and inter-assay variation as well as variation between the results obtained in different laboratories (Table 6). Theoretically, a normal RP value would be less than 10%, considering that the majority of the RNA is degraded within 24 h, and that platelets have a normal mean lifespan of 7 to 10 days (Ingram & Coopersmith, 1969; Ault et al, 1995; Dale et al, 1995). In healthy controls, Bonan et al (1993) have demonstrated a difference in RP percentage between healthy males and females, the proportion of RP being higher among women, although such a sex-related difference has never subsequently been confirmed (Kienast & Schmitz, 1990; Watanabe et al, 1995).

Table 6. Different ways of estimating the degree of fluorescence of TO-stained platelets (reticulated platelets): control samples and ways of setting the threshold for positivity.

Sample	Analysis of TO fluorescence*	Threshold for positivity (control)	Controls			Reference
			n	RP (%) Mean±SD	RP range (%)	
Blood	FL histogram	unstained sample, normal 1%	50	8.6±2.8	2.8-15.8	Kienast & Schmitz, 1990
PRP	FSC vs. FL plot	RNAase treatment, normal 1% †	69	0.9±0.1		Ault et al, 1992
PRP	FL histogram	PE, erythrocyte control	14 male 15 female	3.64±2.10 5.79±2.22		Bonan et al, 1993
PRP	FL histogram	normal 1%, mean+2SD	40	2.9±2.2		Rinder et al, 1993
PRP	FL histogram	normal 1%, mean+2SD	31 pregnant,1st 22, 2nd 29, 3rd trimester	4.9±4.2 2.5±1.7 5.0±4.2		Rinder et al, 1994
PRP	FL histogram	unstained sample, erythrocyte control	20	4.2±1.9		Romp et al, 1994
PRP	FL histogram	unstained sample, normal 1%	41			Richards & Baglin, 1995
Blood	FSC vs. FL plot	RNAase treatment, normal 1%		0.98±0.41		Watanabe et al, 1995
PRP	FL histogram	PE, erythrocyte control	39 neonates, >36 25, 30-36 25, <30 weeks	4±2.4 4.6±1.7 8.8±5.1	2-12 2-7 3-6	Peterec et al, 1996
Blood	FL histogram	RNAase treatment, PE, normal 2%	22	11.6±4.2		Chavda et al, 1996
Blood	FSC vs. FL plot	normal 1%, PE, unstained sample	40	2.17±0.90		Koike et al, 1998
Blood	FSC vs. FL plot	PE, normal 1%, lyophilized platelets	27	7.9±2.9	2.8-13.9	Saxon et al, 1998

* FL histogram: TO positivity presented as a histogram; FSC vs. FL plot: TO positivity presented as a plot of FSC vs. FL.
† normal n%: ≤n% of TO positivity was considered normal.
Blood: whole blood sample; FL: fluorescence intensity for TO positivity; FSC: forward light scatter; PE: phyco-erythrin-conjugated antibody against platelet glycoprotein to demonstrate platelets; PRP: platelet-rich plasma; RNAase: ribonuclease to demonstrate RNA-specific binding of TO; TO: thiazole orange.

Pregnant women in the third trimester and non-pregnant women have been reported to have similar percentages of RP, in spite of a significant fall in the platelet count in the third trimester of pregnancy (Rinder et al, 1994). The fall in the platelet count has been postulated to be due either to down-regulation of thrombopoiesis or to dilutional aetiology of thrombocytopenia. Significantly higher percentages of RP were reported in four women with pre-eclampsia at 26 gestational weeks, before the onset of any clinical signs (13.9 $\pm$ 11.2% vs. 5.8 $\pm$ 2.2% in controls; Rinder et al, 1994).

Patients with immune thrombocytopenia have been demonstrated to have higher proportions of RP in the circulating blood than controls or patients with thrombocytopenia due to insufficient thrombopoiesis. Kienast & Schmitz (1990) studied thrombocytopenic patients, 21 with a normal bone marrow and 23 with decreased platelet production, and found a significantly higher RP% in the patients with sufficient thrombopoiesis. Ault et al (1992) investigated RP and PA-IgG in 229 patients with thrombocytopenia. Patients with a normal level of PA-IgG had a normal proportion of RP, whereas, in those who were positive for PA-IgG, the absolute number of RP was increased if the thrombocytopenia was moderate (60-100 x 10^9/l) but was normal or decreased if the thrombocytopenia was severe. Richards & Baglin (1995) studied 79 thrombocytopenic patients (25 with immune thrombocytopenia and a normal bone marrow, 31 with bone marrow aplasia, and seven with consumptive coagulopathy) and found that the patients with increased peripheral platelet destruction had a higher RP% than those with decreased platelet production. Similar results have been reported by other groups (Watanabe et al, 1995; Chavda et al, 1996; Peterec et al, 1996; Koike et al, 1998; Saxon et al, 1998; Stohlawetz et al, 1999). It has even been postulated that the RP% alone has an even better predictive value than PA-IgG measured with flow cytometry for the diagnosis of autoimmune thrombocytopenia (Rinder et al, 1993). Thus, TO positivity has been suggested to serve as a marker of an increased proportion of RP.

AIMS OF THE STUDY

The aims of this study were to develop laboratory methods for investigating the factors potentially affecting the pathogenesis of autoimmune thrombocytopenia and to subclassify thrombocytopenic patients into different groups with the following specific objectives:

- to test for the presence of platelet-associated antibodies (PA-IgG) in patients with thrombocytopenia,

- to study the glycoprotein (GP) IIbIIIa and IbIX specificity of the platelet antibodies in patients with PA-IgG,

- to study whether the platelet GP V plays a role as a target antigen in patients with thrombocytopenia,

- to determine the polymorphism H131R of the platelet Fcγ receptor in patients with thrombocytopenia and to study whether the platelet-associated antibodies are associated with platelet Fcγ receptor polymorphism, and

- to evaluate the measurement of reticulated platelets by using thiazole orange (TO) staining and flow cytometry.

MATERIALS AND METHODS

1. ETHICAL ASPECTS

Approval for these studies was obtained from the Ministry of Social Affairs and Health in Finland. The ethical committee of the Finnish Red Cross Blood Transfusion Service accepted the plan of the study.

2. STUDY SUBJECTS

2.1. Patients

Consecutive blood samples that were sent to the Platelet Laboratory at the Finnish Red Cross Blood Transfusion Service from patients with histories of thrombocytopenia for detection of platelet antibodies were included in the studies if sufficient platelets for these additional investigations could be isolated (I-III). A total of 438 samples were investigated (I-IV; Table 7). The diagnoses of the different groups of patients are summarised in Table 8.

Table 7. Material and methods used in different studies.

	Study I	Study II	Study III	Study IV
Patients (n)	159	115+1*	113	50
Controls (n)	495	153	93	54
PIFT†	PA-IgG	PA-IgG	PA-IgG	PA-IgG
MAIPA**	GP IIbIIIa+IbIX+IaIIa, GP V	GP IIbIIIa+IbIX, GP V	GP IIbIIIa+IbIX	
FcγRIIA			FcγRIIA	
RP				RP

* Of the total 882 thrombocytopenic patients screened for PA-IgG, 115 samples with strongly positive PA-IgG (fluorescence intensity ≥400) were included. Additional patient with gold-induced thrombocytopenia was included as a case report. Forty-three (of 115, 37%) were also included in Study III.
† detection of PA-IgG. ** detection of GP-specific PA-IgG.
FcγRIIA: Fcγ receptor type IIA genotyping; GP: glycoprotein; MAIPA: monoclonal antibody-specific immobilization of platelet antigens; PA-IgG: platelet-associated IgG; PIFT: platelet immunofluorescence test;
RP: reticulated platelets detected by flow cytometry

Study I. Samples from 159 patients (mean age 50 years, range 0-87 years; 87 male patients) were collected during a 14-week period (November 1993-March 1994).

Study II. Samples from altogether 882 patients were collected during a 15-month period (July 1995-October 1996). Of these 882 patients, only samples with strong positive PA-IgG (median fluorescence intensity $\geq$400) were included, n=115 (mean age 50 years, range 7-87 years; 45 male patients). In addition, one female patient with seronegative rheumatoid arthritis and gold-induced thrombocytopenia was included in the study as a case report. First, the platelet count was found to be normal (218 x 10^9/l), but seven months after the initiation of gold therapy with altogether 810 mg of sodium aurothiomalate, purpura appeared on the lower extremities and mucosal bleeding started. The platelet count was 65 x 10^9/l with a nadir of 5 x 10^9/l 6 days later. Bone marrow examination and abdominal ultrasound scanning were normal. Sodium aurothiomalate injections were discontinued and prednisone treatment was started, 60 mg daily with declining dosage.

Study III. Samples from 113 patients (mean age 47, range 4-84 years, 55 male patients) were collected during an 11-month period (July 1995-May 1996; with an additional 10 months for Group II). Group I comprised 36 (20 female) patients with strongly positive PA-IgG (median fluorescence intensity $\geq$400) and strongly positive GP IIbIIIa and/or GP IbIX-specific PA-IgG (ratio $\geq$4.0). Group II consisted of 13 (8 female) patients with strongly positive PA-IgG and negative GP IIbIIIa or GP Ib-specific PA-IgG (ratio < 1.5). Group III comprised 64 (30 female) patients with negative PA-IgG (fluorescence intensity < 280) and no demonstrable GP IIbIIIa or Ib-specific PA-IgG (ratio $\leq$1.2).

Study IV. Samples from 72 women patients were sent for detection of platelet antibodies from two hospitals (Department of Obstetrics and Gynecology, Helsinki University Hospital and Helsinki City Maternity Hospital) during a 1-year period (August 1997-July 1998). The criteria for inclusion were that samples were from women with either maternal (platelet count <130 x 10^9/l) or neonatal (platelet count <150 x 10^9/l) thrombocytopenia. Twenty-two patients were excluded because of more complicated medical histories. The remaining 50 patients were subclassified as follows: Group 1A (n=11) consisted of thrombocytopenic pregnant women in the third trimester. Group 1B (n=9) consisted of thrombocytopenic women after full-term pregnancy, samples obtained after delivery. Group 2 (n=30) consisted of healthy mothers after full-term pregnancy with a thrombocytopenic newborn infant.

Table 8. Diagnoses of patients in Studies I-IV

Diagnosis *	Study I	Study II	Study III†			Study IV
			Group I	Group II	Group III	
n	159	115+1**	36	13	64	50
ITP	52	28	9	6	23	
Haematological neoplasia	16	7	4	1	8	
Collagenosis	12	6	4		3	
TP and anaemia		8	4		2	
Infection	10					
Chronic liver disease	9	5		1	1	
Rheumatoid arthritis	7	6	1		2	
gold-induced TP		1**				
Neoplasia with TP	2	7	2			
Drug-induced TP	4	6	1	1	1	
Pancytopenia	3	3	3	2	4	
Inflammatory bowel disease	2	4				
Thrombotic TP	2	2				
Renal disease	6					
Pregnancy with TP	3					11
TP, after full-term pregnancy						9
no TP, newborn with TP						30
Thyroid disease	2					
Miscellaneous TP	17	31	8	2	15	
no TP	12	2			5	

* Patients were compiled from routine samples sent to our laboratory for analysis of platelet antibodies. Diagnoses are based on the clinical data supplied (I-III) or collected from the records (IV).
† Group I: strongly positive platelet-associated IgG (PA-IgG) and positive GP-specific PA-IgG; Group II: strongly positive PA-IgG and no GP-specific PA-IgG; Group III: no PA-IgG or GP-specific PA-IgG.
** Of the total 882 thrombocytopenic patients screened for PA-IgG, samples with strongly positive PA-IgG were included, n=115. Additional patient with gold-induced thrombocytopenia was included as a case report. Forty-three (of 115, 37%) were also included in Study III.
ITP: idiopathic thrombocytopenic purpura; TP: thrombocytopenia

2.2. Controls

Platelet samples from healthy blood donors were used as random controls.

Direct PIFT (I-IV). In each test series we examined a fresh platelet sample and a pooled sample from five donors as negative controls, and a sensitised sample as a positive control. A total of 495 (I) and 153 (II, III) control samples were investigated.

Direct MAIPA (I-III). At least two negative controls and a sensitised positive control were examined in parallel by direct MAIPA in each test series. In GP V-specific direct MAIPA, 18 negative controls and a sample from a patient with strong positive GP V-specific PA-IgG were used as controls (II).

FcγRIIA genotyping (III). The distribution of the FcγRIIA polymorphism was determined in 93 (45 female) donors.

Reticulated platelets (IV). Samples from 54 donors (mean age 39 years) were investigated: 38 were male (70 %; age range 24-66 years) and 16 female (range 27-51 years). At least one control sample was used in each test series.

3. METHODS

3.1. Platelet preparation and storage

Blood samples from patients (27 ml of venous blood anticoagulated with 3 ml of liquid 5% Na_2EDTA in phosphate-buffered saline (PBS) in plastic tubes (Falcon; Becton Dickinson, Lincoln Park, New Jersey) were sent overnight to our laboratory. Control samples consisted of 10 ml of blood. Platelets were isolated by differential centrifugation, and the platelet suspension was washed three times with a solution of PBS containing 0.2% bovine serum albumin (BSA). Platelets were fixed in a solution of 1% paraformaldehyde in PBS for 5 min at room temperature (RT), and then washed twice. The platelet concentration was determined with an electric cell counter (Medonic, Solna, Sweden). The final concentration of the washed platelet suspension was adjusted to 500 x 10^9/l for direct PIFT and for analysis of reticulated platelets. For direct MAIPA, the platelets were washed once more and suspended in physiological NaCl to a final platelet concentration of 1000 x 10^9/l. The platelets were stored for no longer than 2 weeks at +4 ^oC (I) or at -70 ^oC (II, III).

3.2. Detection of PA-IgG and GP-specific PA-IgG

Platelet-associated IgG (PA-IgG) and GP-specific PA-IgG were detected with the direct PIFT (I-IV) and the direct monoclonal antibody-specific immobilisation of platelet antigens (MAIPA; I-III), respectively.

3.2.1. Direct platelet immunofluorescence test (Direct PIFT)

PA-IgG was measured by direct PIFT (von dem Borne et al, 1978). Freshly prepared platelet suspension

(500 x 10^9/l) containing a total of 50 x 10^6 platelets, was incubated with 100 µl of diluted fluoro-isothiocyanate-conjugated rabbit anti-human IgG, specific for the Fc fragment (DAKO, F-123; Dakopatts A/S, Copenhagen, Denmark). After incubation for 30 min at 37 °C on microtitre plates precoated with BSA, the platelets were washed once with 1% BSA in Na-phosphate EDTA-buffer solution. The platelets were then diluted in 1 ml of buffer for analysis with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 15.2 mW air-cooled 488 nm argon-ion laser. Logarithmic amplification was used on the FSC, side light scatter and fluorescence intensity (FL). Data of 2 000 ungated events were analysed with a software program (Lysys II, Becton Dickinson). In each test series, an FSC versus side light scatter dot plot was used to draw a gate containing the majority of the platelet population of the control sample. The median value of the FL was used to determine the value for PA-IgG. In each test series we examined a fresh platelet sample from one healthy blood donor as a random negative control (n=495; mean FL 221, median 217, SD 47, CI 217-225) during over a 4-year period (I), and (n=153, mean FL 212, median 210, SD 39, CI 205-218, CV 18.4%) during a 15-month period (II). The control values were normally distributed (Shapiro W test, $P<0.0001$). A pooled sample from five healthy blood donors serving as a negative control (median 186, CV 5.9%), and a sensitised platelet sample as a positive control (median 605, CV 1.4%) were also examined in each test series. Quality control of the instrument was carried out in each test series using standard labelled (median 790, CV 1.0%) and unlabelled (median 315, CV 1.8%) fluorescent beads (CaliBRITE Beads, Becton Dickinson). In Study I, an FL of 300 (mean + 2 SD) was taken as an arbitrary cut-off value above which sample was positive. In Study II, a sample was considered positive when the median FL was $\geq$300 and strongly positive when $\geq$400.

3.2.2. Direct monoclonal antibody-specific immobilisation of platelet antigens (Direct MAIPA)

The direct MAIPA (Kiefel et al, 1991) was modified. The following monoclonal antibodies were used: AP1, specific against the GP Ibα (Okita et al, 1985; a generous gift from Dr R Montgomery, Milwaukee, WI, USA); AP2, specific against the GP IIbIIIa complex (Pidard et al, 1983; a generous gift from Dr R Montgomery, Milwaukee); Gi9, specific against the GP IaIIa purchased from Immunotech (Marseille, France); SW16, specific against the GP V (Modderman et al, 1992; purchased from the Central Laboratory of the Netherlands Red Cross, Amsterdam, Netherlands).

Study I. The platelet suspension (1000 x 10^9/l) of 60 µl was washed once with 0.9% NaCl and resuspended in PBS containing 2% BSA. Aliquots of 0.2 µg of each of the three monoclonal antibodies, AP2, AP1 and Gi9, were applied simultaneously to the platelets and incubated for 30 min at 37 ^oC. For separate measurements of GP Ib- and GP V-specific antibodies, 0.2 µg of monoclonal antibody AP1 or SW16 was added to 25 µl or 40 µl, respectively, of the platelet suspension. After washing three times with 0.9% NaCl, the platelets were solubilised in detergent (0.5% Triton X-100, 1.21 g/l Tris in isotonic saline) and incubated for 30 min at +4 ^oC. The platelet lysates were centrifuged at 13 000 rpm for 15 min, and 50 µl of the supernatant was transferred to tubes containing 200 µl Tris-buffered saline (TBS) in which the samples were stored overnight at +4 ^oC. The wells of a microtitre plate (A/S Nunc, Roskilde, Denmark) were coated with goat polyclonal anti-mouse IgG (Jackson Immunoresearch Inc., West Grove, PA) diluted 1:500 with a coating buffer (0.015 M $NaCO_3$, 0.035 M $NaHCO_3$, pH 9.6, containing 0.02% NaN_3) in triplicate, washed six times with an automatic microtitre plate washer (MultiWash, Tri-Continent Scientific, Grass Valley, CA), and blocked for 30 min at RT with TBS before addition of 70 µl of lysate to each well. After incubation for 90 min at +4 ^oC, the wells were washed six times, and 100 µl of peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:2000 with TBS, was added. After incubation for 120 min at +4 ^oC, the wells were washed six times with TBS. Substrate solution (1,2-phenylene-diamine, A/S DAKO, Denmark in citric-phosphate buffer) was added, and after incubation for 30 min at RT, the absorbance was measured at 492 nm (Multiskan, Labsystem Ltd, Helsinki, Finland). The patient's absorbance value was divided by the mean negative control absorbance of each test series to obtain the relative absorbance. A sample was considered positive when the relative absorbance was $\geq$1.5.

Study II and III. Frozen platelet suspensions (1000 x 10^9/l) of 25-µl aliquots were quickly thawed and incubated with 0.2 µg of the monoclonal antibodies AP1 and AP2. For separate measurement of GP V-specific PA-IgG, 0.2 µg of the monoclonal antibody SW16 was added to 80 µl of the platelet suspension. Here, for the relative absorbance (patient's absorbance value divided by mean negative control absorbance of each test series), the term 'ratio' was used (same formula). A sample was considered positive when the ratio was $\geq$1.5, and strongly positive when >4.0 (III) and $\geq$6.0 (II) . In the GP IIbIIIa- and/or GP IbIX-specific direct MAIPA (II), negative and positive controls had interassay CVs for ratios 11% (median 1.1) and 12.5% (median 22.0), respectively.

Separately, 41 paired fresh and frozen samples were tested for GP IIbIIIa- and/or GP IbIX-specific PA-IgG to check whether freezing affected the results. The ratios for the fresh and frozen samples (mean 1.6, SD 1.7, range 0.9-11.7 and mean 1.6, SD 1.6, range 0.8-10.7, respectively) correlated well (Spearman R=0.82, P<0.001). Samples from healthy blood donors (n=18) were used as negative controls in GP V-specific direct MAIPA (median 1.0, range 0.9-1.0, CV 3.7%). The linearity of the method was determined with a strong positive GP V-specific PA-IgG sample at successive platelet dilutions (Spearman R=0.99, range of ratio 2.1-21.4).

3.3. Genotyping of the FcγRIIA

The genomic DNA of the leukocytes from buffy coats (extracted from EDTA-anticoagulated blood), stored frozen at -20 ^{o}C, was isolated by a salting-out procedure (Miller et al, 1988). An FcγRIIA-gene-specific fragment of approximately 1000 bp was first amplified according to Osborne et al (1994) with modifications. PCR was performed by adding about 500 ng of the purified genomic DNA template to 50 µl of reaction mixture containing 25 pmol of each oligonucleotide primer, 5 µl of 10X Dynazyme buffer, 2 U of DNA Polymerase (Dynazyme, Finnzymes, Espoo, Finland), and 5 µl of 2.5 mM polymerisation mix (Pharmacia LKB Biotechnology, Upsala, Sweden). The steps in the PCR analysis for the thermal cycler (DNA engine, MJ Research, Inc., Watertown, Massachusetts) were 3 min denaturation at 96 ^{o}C, amplification for 30 cycles of 1 min at 96 ^{o}C, 1 min at 58 ^{o}C, and 1 min at 72 ^{o}C. Then 5 µl of the product obtained by PCR was analysed by electrophoresis on 1.5% agarose gel. The genomic FcγRIIA 494-G/A polymorphism does not lead to formation or loss of a restriction site. Therefore, a nested PCR was performed using the FcγRIIA-specific product as a PCR template according to de Haas et al (1995). The primers create a novel restriction site for *BstUI* (New England Biolabs, Beverly, MA) in cases in which G was present at position 494. For the *BstUI* digestion, 8 µl of amplified DNA product of 134 bp obtained from the second PCR was mixed with 4.5 µl of sterilised water, 1.5 µl of the 10X buffer supplied with the restriction enzyme, and 1U of the restriction enzyme *BstUI.* After incubation overnight, the fragments were analysed on a 3% agarose (NuSieve; FMC, Rockland, ME) gel with a molecular weight standard (Fig 5). If there was a G at position 494, the digestion resulted in two fragments of 115 bp and 19 bp. Samples repeatedly found to be of known genotypes were analysed in each test series.

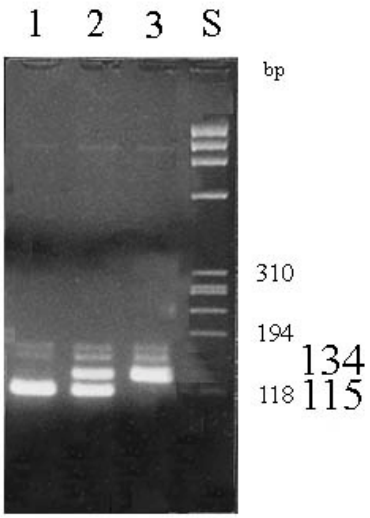

Fig 5. *BstUI* restriction analysis of the 134-bp FcγRIIA-specific fragment containing the polymorphic nucleotide 494. A 1.0-kb FcγRIIA -specific fragment was amplified from genomic DNA, followed by nested PCR. With mismatch the primers of the nested PCR created a *BstUI* restriction site in the R131 (494G) allele only. Homozygous RR131 fragments were digested, resulting in bands 115 bp and 19 bp (not clearly visible; lane1). In heterozygous HR131, two bands (134 bp and 115 bp) were visible (lane 2). Homozygous HH131 fragments were not digested (lane 3).

3.4. Measurement and data analysis of platelets stained with TO (RP)

For the TO staining, the published procedures were modified (Ault et al, 1992; Bonan et al, 1993; Rinder et al, 1993; Richards & Baglin, 1995). Samples were analysed within 48 hours. A paraformaldehyde-fixed platelet suspension (500 x 10^9/l) of 10 µl was incubated with 90 µl of TO solution (RetiCount, Becton Dickinson, San Jose, CA). After incubation for 60 min at RT in the dark, 400 µl of buffer was added, and the platelets were analysed in a FACScan flow cytometer. For the first 21 series (29 patients and 23 controls), data of 5 000 ungated events were collected and analysed with a Cellquest™ Software program (Becton Dickinson, San Jose, CA). In subsequent series, the number of events was increased to 50 000. Logarithmic amplification of FSC, side light scatter and FL were used. FSC was plotted against side light scatter, and this dot plot was used to draw a gate containing the majority of the platelets (approximately 99%) in each test series. This gating was used to analyse the samples of that particular series.

Data analysis methods were based on two principles (Fig 6):

A. Patient median FL1. The values of FL and FSC for the patients' samples and control samples were plotted as histograms. Median values of FSC were obtained, and median FLs were also obtained for the control and for the patients' samples, Control median FL1 and Patient median FL1, respectively.

B. Relative FL1. To compare the results of the separate test series, a Relative FL1 was obtained for every patient's sample, using the formula: Relative FL1 = [(Patient median FL1 - Control median FL1): Control median FL1] x 100.

C. % RP. A dot plot of FL vs. FSC was made for every patient sample and control sample. The dot plot of the control sample was used in the analysis settings. A line was drawn at the edge of the platelet cluster corresponding to the slope of the platelet population. This slope line was adjusted to segregate 94.0-95.9% of the events below the line (TO-negative). This setting was used to analyse the patients' samples for that particular series.

Ribonuclease (RNAase) treatment. To demonstrate the specificity of the TO staining of platelet RNA, random platelet samples were treated with RNAase before the staining (Ribonuclease A, 50-100 Kunitz units/mg, Sigma Chemical CO, St. Louis, MO). RNAase (5 µl; 1 mg/100 ml) was used to treat 20 µl of platelet suspension for 30 min at +37 ^{o}C. The TO positivity (median FL1) was diminished in all the patients tested (n=16; mean decrease 8.0%, CI 5.7-10.3 %) and control samples (n=5; mean 4.7%, CI 0.7-8.7%).

3.5. Statistical analysis

The mean, median, SD, range and 95% confidence intervals (CI) were calculated to provide estimates (I-IV). Correlations were calculated with the Spearman rank order correlation (I, II, IV). Differences between populations were calculated with the Mann-Whitney U test or the Student's t test, taking $P<0.05$ as significant (I-IV). The Shapiro W test was used to evaluate a normal distribution (I). The coefficients of variation (CV) were calculated for the methods (II). The distribution of FcγRIIA genotypes and allele frequencies in patients and controls were compared by the exact 2 x k test (III). Fisher's exact test was used for comparing the age profiles between the groups of patients with different FcγRIIA genotypes (III). In the statistical analysis, the software used comprised InStat (GraphPAD Software), StatXact 3 (CYTEL Software Corporation) and Statistica for Windows (StatSoft inc.).

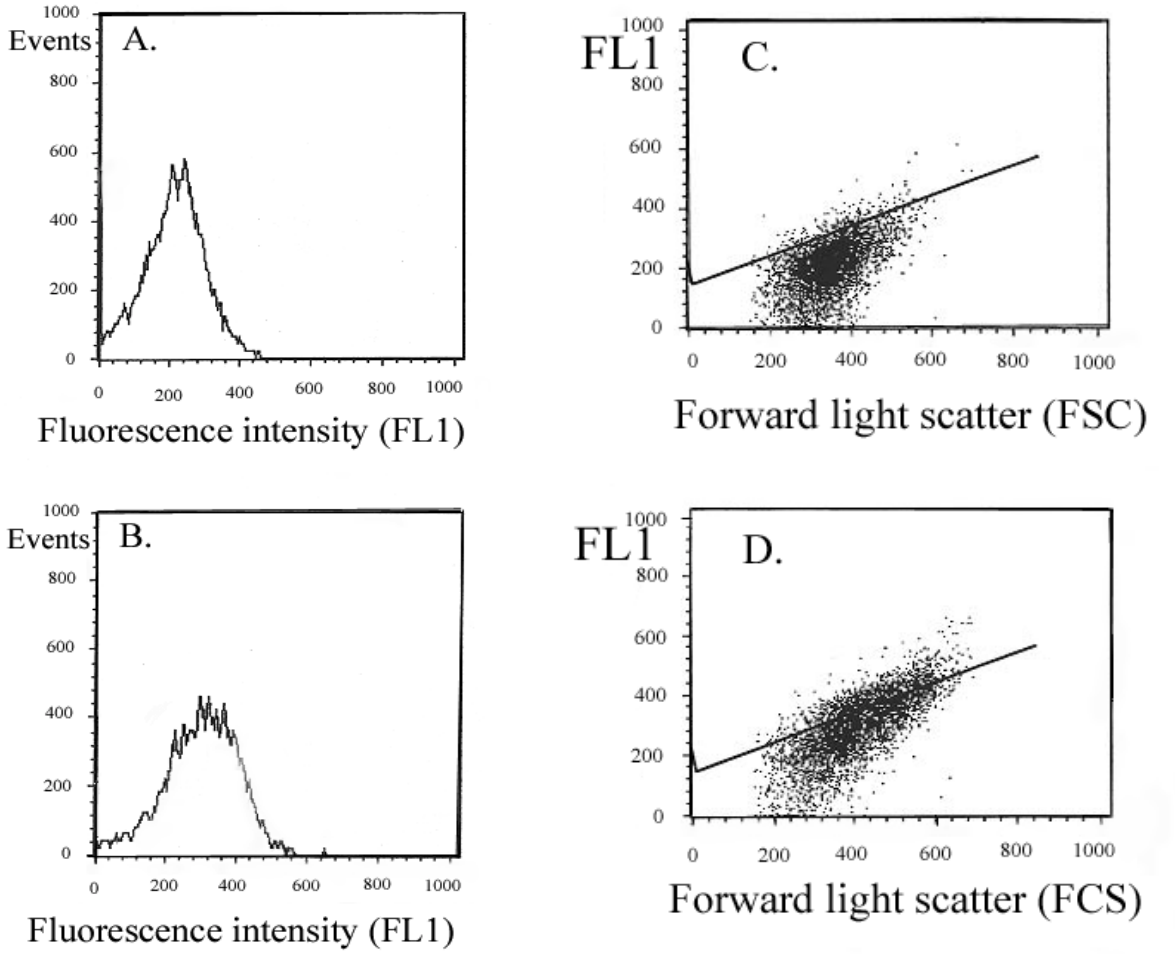

Fig 6. Different ways of estimating the degree of fluorescence of thiazole orange (TO)-stained platelets in a control sample and in a patient's sample considered to be TO-positive. The fluorescence intensities were plotted as histograms and median values were obtained for every control (A; Control median FL1 213) and patient samples (B; Patient median FL1 304). The Relative FL1 for the patient's sample was calculated: Relative FL1 = [(Patient median FL1-Control median FL1): Control median FL1] x 100 (here, Relative FL1 42.7%). For the control sample, the FSC versus FL1 scatter (% RP; C) was plotted. A sloping line segregating 95% of the events below the line was set parallel to the platelet cluster, here leaving 4.3% of the events above the line. This setting was also used in the analysis of the patient's sample (D) in that particular test series. The % RP for the patient sample was 26.1%.

RESULTS

1. SREENING FOR PA-IgG IN THROMBOCYTOPENIC PATIENTS (I-IV)

Study I. Direct PIFT for measuring PA-IgG on patient's platelets was considered to be positive (an arbitrary cut-off value of 300 for median fluorescence intensity) in 53% (84/159) of the patients (Fig 7). Strongly positive PA-IgG (median fluorescence intensity ≥400) was found in 25 (16%) patients (Fig 8).

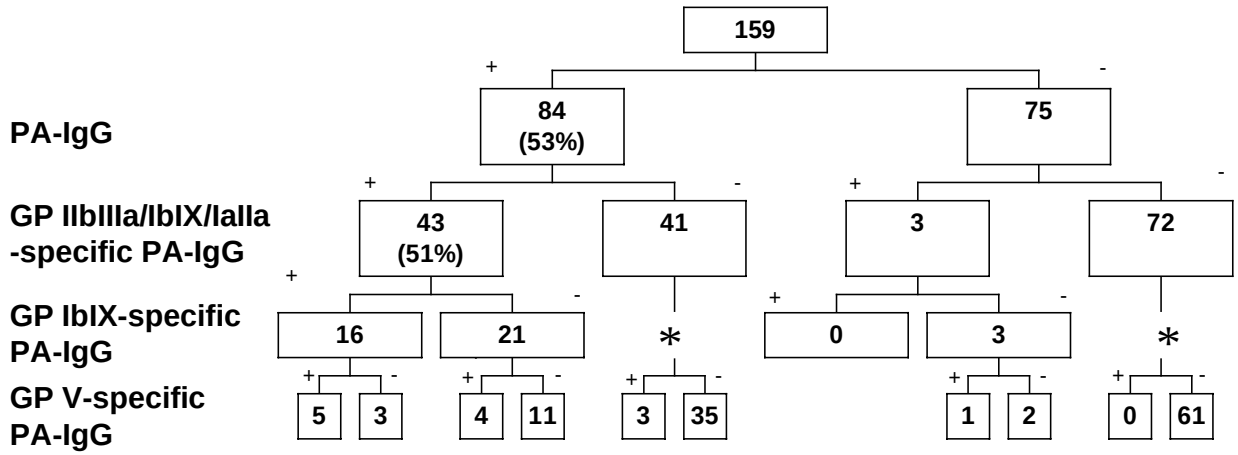

Fig 7. Results of the direct PIFT for platelet-associated IgG (PA-IgG) and the direct MAIPA for GP-specific PA-IgG in Study I. * Samples were not studied for GP IbIX-specific PA-IgG.

Study II. Direct PIFT for measuring PA-IgG was positive (median fluorescence intensity ≥300) in 41% (366/882) of the patients. Strongly positive PA-IgG (median fluorescence intensity ≥400) was found in 115 (13%) patients (Fig 8). One additional patient with gold-induced thrombocytopenia (presented as a case report) had strongly positive PA-IgG (fluorescence intensity 503).

Study III. Group I (n=36) and Group II (n=13) consisted of samples from patients with strongly positive PA-IgG (median fluorescence intensities ≥400). The samples in Group I were also positive for GP-specific PA-IgG, whereas those in Group II had no GP-specific PA-IgG. PA-IgG was shown to be higher in Group I than in Group II (mean fluorescence intensity 524, SD 112 vs. mean 433, SD 21 Mann-Whitney U test, P=0.003), indicating that the antibody profiles of the two groups differed significantly from each other.

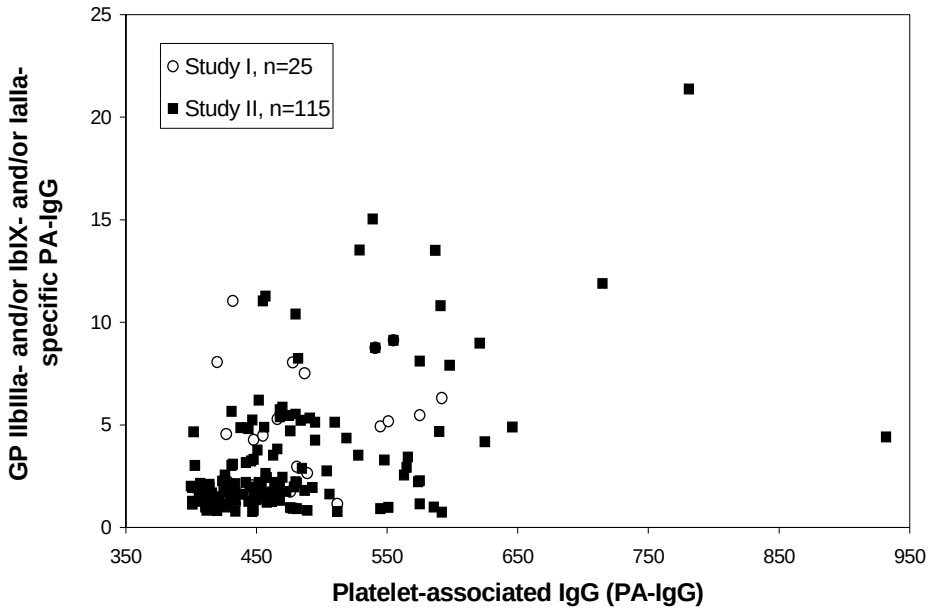

Fig 8. Comparison between the median fluorescence intensities for PA-IgG measured by the direct PIFT and the ratios for glycoprotein (GP)-specific PA-IgG measured by the direct MAIPA in 140 patients with fluorescence intensities ≥400. Combined results of Studies I and II.

Study IV. Altogether 50 patients were examined, and PA-IgG was positive (median fluorescence intensity ≥300) in 4 of 11 pregnant women with thrombocytopenia (Group 1A), in 3 of 9 thrombocytopenic women after full-term pregnancy (Group 1B) and in 3 of 30 healthy women with a thrombocytopenic newborn infant (Group 2). PA-IgG was shown to be higher in Group 1A than in Group 2 (mean fluorescence intensity 293, median 289, SD 57, CI 255-331 vs. mean 235, median 226, SD 54, CI 215-255; Mann-Whitney U test, P=0.003).

2. GP-SPECIFIC PA-IgG (I-II)

Study I. Of the patients who were positive for PA-IgG, 51% (43/84) were also positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG (Fig 7). In addition, 2% of the patients (3/159) with no PA-IgG were weakly positive for GP-specific PA-IgG (range of ratio 1.5-1.9), suggesting that the PIFT was a sensitive method and suitable as a reference method. Of all the patients, 29% (46/159) were positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG (Fig 7).

The correlation between the fluorescence intensities for PA-IgG and the relative absorbances for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG was significant (Spearman R=0.79, P<0.001; Fig 8). The fluorescence intensities for PA-IgG in the patients who were positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG were higher than in those with no GP-specific PA-IgG (n=46, mean 411, median 395, SD 81, CI 387-435 vs. n=113, mean 284, median 272, SD 59, CI 273-294; Mann-Whitney U test P<0.001). Among the patients who were positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG, the fluorescence intensities for PA-IgG were higher in the female (n=18) than in the male (n=28) patients (mean 451, median 452, SD 76, CI 413-489 vs. mean 386, median 365, SD 68, CI 357-414; Mann-Whitney U test, P=0.004).

Study II. GP IIbIIIa- and/or GP IbIX- specific PA-IgG were found in 20% (176/882) of the patients. GP IIbIIIa- and/or GP IbIX-specific PA-IgG was positive in 83% (95/115) of the patients who were strongly positive for PA-IgG. Of the 366 patients who were positive for PA-IgG, 206 (56%) were also positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG. In addition, 16 patients (2% of 882 patients) with no PA-IgG were weakly positive for GP-specific PA-IgG. In the study population of 882 patients, the correlation between the fluorescence intensities for PA-IgG and the ratios for GP IIbIIIa- and/or GP IbIX- specific PA-IgG was significant (Spearman R=0.64, P<0.001), as expected from the results of Study I.

Patients with the most strongly positive responses to both PA-IgG and GP-specific PA-IgG seemed to have thrombocytopenia of suggested autoimmune origin (I, II; Table 9).

3. GP V-SPECIFIC PA-IgG (I-II)

Study I. Positive GP V-specific PA-IgG was demonstrated for 13 (10%) patients of the 125 whose samples were studied (Fig 7). Of these 13 patients, 12 were positive for PA-IgG by direct PIFT, of whom seven were strongly positive. Ten patients were also positive for GP IIbIIIa- and/or GP IbIX- and/or GP IaIIa-specific PA-IgG (Table 9). GP V-specific PA-IgG was detectable even in the samples with no demonstrable GP IbIX-specific PA-IgG (5 patients; Fig 7), suggesting a separate sole target role of GP V. However, samples in which both GP V-specific and GP IbIX-specific PA-IgG were detected showed a significant correlation between the relative absorbances (R=0.82).

Table 9. Thrombocytopenic patients with strongly positive platelet-associated IgG (PA-IgG) and positive glycoprotein V (GP V)-specific PA-IgG in Studies I and II.

Patient	Study	Sex	Age	Diagnosis	PA-IgG*	GP IIbIIIa and/or IbIX and/or IaIIa PA-IgG†	GP V PA-IgG†
1	II	f	58	SS with gold therapy	646	4.9	21.8
2	I	m	43	RA with gold therapy, TP	592	6.3	20.1
3	I	f	69	Liver disease, TP	575	5.5	12.2
4	II	m	20	ITP, AIHA	566	3.4	15.3
5	II	f	69	RA with gold therapy, TP	565	2.9	22.4
6	I	f	50	RA with gold therapy, TP	545	4.9	3.7
7	I	m	69	Quinidine therapy	541	8.8	11.7
8	II	f	31	ITP	528	3.5	7.8
9	II	f	13	Anaemia, TP	519	4.4	4.8
10	II	m	37	ITP	491	5.3	2.0
11	I	m	60	ITP, AIHA	487	7.5	3.9
12	II	f	44	Thrombosis, TP	463	3.5	1.9
13	II	f	76	Quinidine-induced TP	462	1.3	7.7
14	II	f	25	TP	457	2.6	2.7
15	II	m	59	RA	454	2.0	2.0
16	II	f	65	SLE	451	3.8	1.9
17	I	f	28	Glomerulonephritis, TP	448	4.3	3.5
18	II	f	31	Anaemia, TP	446	3.2	2.8
19	II	m	19	ITP	442	3.2	12.4
20	II	m	51	TP	424	2.3	14.0
21	II	m	37	Liver disease, TP	422	1.5	1.5
22	I	f	3	TP	415	1.2	1.8
case**	II	f	54	RA, gold-induced TP	503	2.5	18.6

* Median fluorescence intensities for PA-IgG ≥400 by PIFT were considered strongly positive.
† Ratios for GP specific PA-IgG ≥1.5 by MAIPA were considered positive. The samples were studied when fresh (I) or after freezing (II). ** Additional patient included as a case report.
Patients with strongly positive PA-IgG by direct PIFT and positive GP V-specific PA-IgG by direct MAIPA were included. AIHA: autoimmune haemolytic anaemia; ITP: idiopathic thrombocytopenic purpura; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SS: Sjögren`s syndrome; TP: thrombocytopenia

Study II. Positive GP V-specific PA-IgG was found in 15 (22%) of the study population's 69 samples (Table 9). In seven samples, the GP V-specific PA-IgG was considered strongly positive (ratio ≥6.0). Thus, a positive GP V-specific PA-IgG partly explained (15/69, 22%) the strongly positive PA-IgG.

One patient with strongly positive PA-IgG and strongly positive GP V-specific PA-IgG had no GP IIbIIIa- or GP IbIX-specific PA-IgG (Table 9, Patient 13). Strongly positive GP V-specific PA-IgG was found in seven samples without strongly positive GP IIbIIIa- and/or GP IbIX-specific PA-IgG. Of the 46

samples with strongly positive PA-IgG but without sufficient platelets (i.e. which could not be studied for GP V-specific PA-IgG), 13 were strongly positive for GP IIbIIIa- and/or GP IbIX-specific PA-IgG. The ratios for GP IIbIIIa- and/or IbIX-specific PA-IgG and those for GP V-specific PA-IgG were directly correlated (Spearman $R=0.53$, $P<0.001$).

One additional patient with gold-induced thrombocytopenia had initially strongly positive GP V-specific PA-IgG (ratio 18.6; presented as a case report, Table 9). Two additional samples obtained during recovery from thrombocytopenia in the following 3 months showed that strongly positive GP V-specific PA-IgG was still present but declining.

4. POLYMORPHISM OF THE PLATELET FCγRIIA H131R (III)

Among the 93 Finnish blood donors tested, the genotypes HH131, HR131 and RR131 were found in 25 (27%), 55 (59%) and 13 (14%), respectively, and thus the frequency of the H131 allele was 0.56 and that of R131 was 0.44 (CI: 0.37-0.51). The distribution of the FcγRIIA alleles of Finnish blood donors did not differ significantly from that of other Caucasians (Table 10).

Nor did the frequencies of H131R polymorphism of FcγRIIA differ significantly among the three sets of patients grouped according to their PA-IgG profiles (altogether 113 patients). Furthermore, the groups did not differ significantly from the controls (allelic frequencies in Table 11). However, a trend towards a higher frequency of genotype RR131 of FcγRIIA in patients with no detectable PA-IgG (Group III) was observed (Exact 2 x k test, $P=0.10$). When the fluorescence intensities for PA-IgG in the patients were grouped according to the FcγRIIA genotypes, those with the genotype HH131 showed a tendency to have higher intensities than those with RR131 (Mann-Whitney U test, $P=0.082$). The genotype RR131 was found in 26 of 113 (23%) patients compared with 13 of 93 (14%) controls. Interestingly, the female patients with the genotype RR131 (n=15) were younger than those with the genotype HH131 (Mann-Whitney U test, $P=0.065$). Among the female patients, there were significantly more under 40 years old in the RR131 group than in the HH131 group (Fisher's exact test, $P=0.006$). Within the RR131 group, the female patients were remarkably younger than the male patients (median age 29 vs. 61 years; Mann-Whitney U test, $P=0.002$).

Table 10. Frequencies of the Fcγ receptor type IIA (FcγRIIA) alleles in different populations.

Population	H131	R131	CI for R131	n	Reference
Finnish	0.56	0.44	0.37-0.51	93	(III)
Caucasian			0.46-0.50	(1947)	
Caucasian	0.47	0.53		264	Denomme et al, 1997
Caucasian	0.45	0.55		259	Botto et al, 1996
Caucasian	0.54	0.46		256	Carlsson et al, 1998
Caucasian	0.56	0.44		218	Bachelot-Loza et al, 1998
Caucasian	0.51	0.49		187	Manger et al, 1998
Caucasian	0.59	0.41		167	Bachelot et al, 1995
Dutch	0.53	0.47		123	Reilly et al, 1994
Caucasian	0.47	0.53		102	Arepally et al, 1997
European	0.58	0.42		69	Duits et al, 1995
British	0.47	0.53		66	Smyth et al, 1997
Caucasian	0.53	0.47		61	Williams et al, 1998
Greek	0.62	0.38		52	Smyth et al, 1997
American	0.55	0.45		47	Sanders et al, 1994
Caucasian	0.50	0.50		41	Atsumi et al, 1998
Caucasian	0.50	0.50		35	Osborne et al, 1994
Asian			0.22-0.36	(206)	
Japanese	0.78	0.22		105	Kobayashi et al, 1997
Chinese	0.69	0.31		49	Botto et al, 1996
Japanese	0.77	0.23		18	Osborne et al, 1994
Chinese	0.72	0.28		18	Osborne et al, 1994
Indian	0.39	0.61		16	Osborne et al, 1994
African			0.46-0.58	(277)	
African-American	0.52	0.48		100	Salmon et al, 1996
Afro-Caribbean	0.45	0.55		77	Botto et al, 1996
African-American	0.44	0.56		50	Norris et al, 1996
African-American	0.44	0.56		50	Sanders et al, 1994

Table 11. Fcγ receptor type IIA (FcγRIIA) allelic frequencies in patients and in controls.

	n	H131 (n)	R131 (n)	CI for R131
Group I*	36	0.58 (42)	0.42 (30)	0.31-0.53
Group II†	13	0.42 (11)	0.58 (15)	0.39-0.77
Group III**	64	0.48 (61)	0.52 (67)	0.44-0.60
Controls	93	0.56 (105)	0.44 (81)	0.37-0.51

* Strongly positive platelet-associated IgG (PA-IgG; fluorescence intensity ≥400) and positive GP-specific PA-IgG (relative absorbance >4.0).
† Strongly positive PA-IgG and no GP-specific PA-IgG (relative absorbance <1.5).
** No PA-IgG (fluorescence intensity <280) or GP-specific PA-IgG (relative absorbance <1.2).

5. RETICULATED PLATELETS (IV)

In 50 patients, fluorescence for TO positivity was estimated in three different ways: Patient median FL1, Relative FL1 and % RP (Fig 9; Table 12). The Patient median FL1 of pregnant women with thrombocytopenia (Group 1A) was significantly higher than that of healthy women with a thrombocytopenic newborn infant (Group 2; mean 306, SD 40, CI 279-332 vs. mean 266, SD 30, CI 255-277; Mann-Whitney U test P=0.004; Table 12, Fig 10), suggesting that RP reflect increased platelet production and features of pathological thrombocytopenic conditions. The patients in Group 1A had stronger Patient median FL1 than the female controls (Mann-Whitney U test, P<0.001), whereas there was no such difference between the patients of Group 1B (thrombocytopenic women after full-term pregnancy) or Group 2 and the female controls. In Group 1A, a significant correlation was observed between the Patient median FL1 and Patient median FSC (Spearman R= 0.62, P=0.043) but in the other groups we found no correlation.

The Patient median FL1 was higher in the 50 detected patients than in the female controls (mean 277, SD 36, CI 266-287 vs. mean 249, SD 34, CI 231-268; Mann-Whitney U test, P=0.015). The correlation between the Patient median FL1 and Patient median FSC was significant (Spearman R=0.33; P=0.021), whereas no correlation was observed between the Control median FL1 and Control median FSC (Spearman R=0.21, P>0.05).

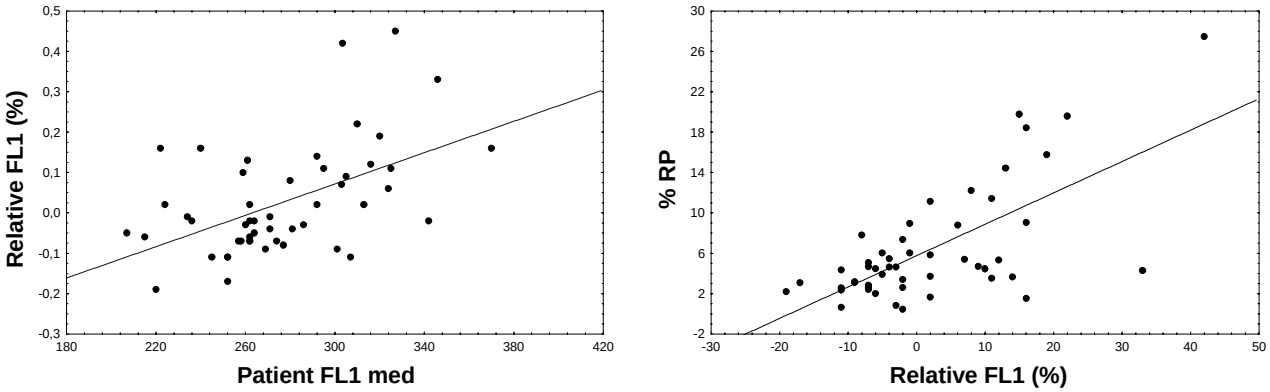

Fig 9. Comparison of different ways of estimating the degree of fluorescence (FL) of platelets stained with thiazole orange (TO) in 50 patients. A. The fluorescence intensities were plotted as histograms, and a median value was obtained for every patient's sample (Patient median FL1). The Relative FL1 for the patient's sample was calculated: Relative FL1 = [(Patient median FL1-Control median FL1): Control median FL1] x 100 in each test series. B. The forward light scatter versus the FL1 plot of a control sample was used to obtain % RP (reticulated platelets) for a patient's sample.

Pregnant women with thrombocytopenia (Group 1A) also had higher levels of PA-IgG than healthy women with a thrombocytopenic newborn (Group 2; Mann-Whitney U test, P=0.003; Table 12). Fluorescence intensities for PA-IgG and Patient median FL1 for RP correlated significantly (Spearman R=0.30, P=0.032).

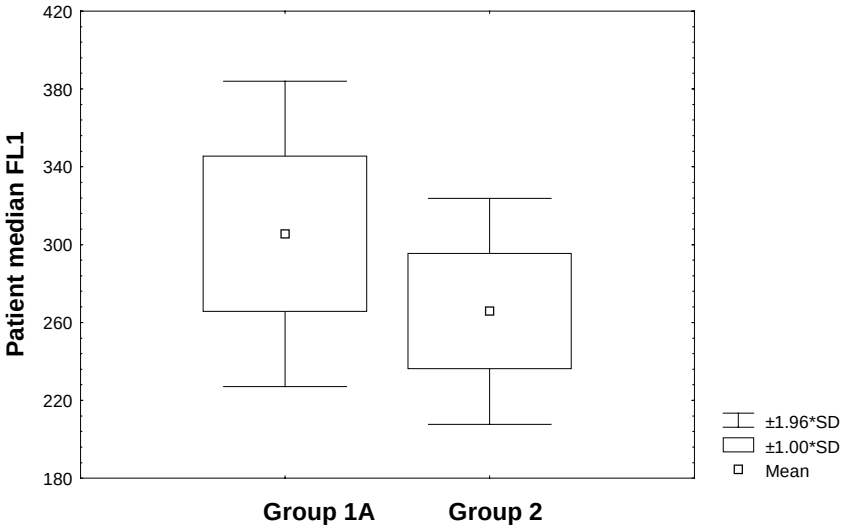

Fig 10. Comparison of the thiazole orange (TO) positivity (Patient median FL1) in two groups of patients: 11 pregnant women with thrombocytopenia (Group 1A) and 30 healthy mothers with a thrombocytopenic newborn infant (Group 2). The difference was significant (Mann-Whitney U test, P=0.004).

Table 12. Different ways of estimating the degree of fluorescence of thiazole orange (TO) stained platelets and platelet-associated IgG (PA-IgG) in patients and controls.

		Thrombocytopenic women		Healthy women	
		during pregnancy Group 1A	after delivery Group 1B	newborn with TP Group 2	Controls
	n	11	9	30	54
median FL1	mean	306*	278	266	267
	median	304	292	262	271
	SD	40	35	30	37
	CI	279;332	251;304	255;277	257;277
	range	236;370	215;325	207;324	192;350
Relative FL1 (%)	mean	12†	3.8	-1.1	
	median	2	7	-4.5	
	SD	20	8.5	11	
	CI	-1.4;25	-2.8;10	-5.1;2.9	
	range	-9;45	-11;14	-19;22	
% RP	mean	7.1	3.9	6.8	
	median	4.3	4.4	5.3	
	SD	8.6	1.1	5.1	
	CI	1.4;12.9	3.1;4.8	4.9;8.8	
	range	0.4;27	1.6;5.4	0.6;19.6	
FSC	mean	447**	421††	388	383
	median	441	422	391	388
	SD	47	37	21	25
	CI	416;479	392;449	380;396	376;390
	range	395;537	368;486	336;421	308;433
PA-IgG	mean	293***	257	235	
	median	289	229	226	
	SD	57	65	54	
	CI	255;331	207;307	215;255	
	range	227;400	187;362	165;405	

* Group 1A vs. Group 2: P=0.004 and Group 1A vs. Controls: P=0.006 by the Mann-Whitney U test.
† Group 1A vs. Group 2: P=0.037.
** Group 1A vs. Group 2: P<0.001; Group 1A vs. Controls P<0.001; †† Group 1B vs. Group 2: P=0.01; Group 1B vs. Controls: P=0.005. *** vs. Group 2: P=0.003.
Median FL1: Median fluorescence intensity (FL) of TO stained platelets obtained with FL histogram;
Relative FL1: [(Patient median FL1-Control median FL1) : Control median FL1] x 100;
% RP: TO-positive platelets determined from a forward light scatter (FSC) vs. FL plot using a segregating line (4-6%) in a control sample; FSC: Median FSC of TO stained platelets with FSC histogram; PA-IgG: detected by PIFT, ≥300 was considered positive; TP: thrombocytopenia.

DISCUSSION

1. DETECTION OF PLATELET AUTOANTIBODIES

The direct platelet immunofluorescence test (PIFT) allows sensitive, reproducible and reliable determination of platelet-specific autoantibodies on the platelet surface (von dem Borne et al, 1978 & 1986b; Ault, 1988). Numerous assays have been used for the detection of platelet antibodies in thrombocytopenic patients, but improvement of the specificity and deeper understanding of the assays and of the disease itself will probably enhance agreement about the applications and usefulness of the new methods. Of randomly chosen patients with heterogeneous histories of thrombocytopenia collected at separate periods of time, half the patients were positive for PA-IgG in the direct PIFT (I, II). Only 2% of the patients in the two studies were demonstrated to have GP-specific PA-IgG, while the reference method gave negative results. This may partly be explained by the low cut-off limit for positivity in MAIPA. In comparison, Brighton et al (1996), using MAIPA, found GP-specific PA-IgG in 10% (8/81) of patients with initially diagnosed immune thrombocytopenia and a negative result obtained with a screening method (ELISA). Accordingly, the PIFT for measuring PA-IgG may well be used as a sensitive reference method for screening PA-IgG when detecting platelet autoantibodies in patients with thrombocytopenia due to different causes. Heterogeneous patient groups may be categorised according to the PIFT result. Thus, PA-IgG serves as one device for defining the various forms of thrombocytopenia.

Platelet GP IIbIIIa and GP IbIX appear to be important target autoantigens. GP IIbIIIa-, GP IbIX- or GP IaIIa-specific autoantibodies were found in almost half the patients in whom the direct PIFT for PA-IgG was positive. A positive direct PIFT with no detectable GP-specific PA-IgG may be caused by PA-IgG against other antigens and also by non-specific antibodies on the platelet surface or immunocomplexes bound by the platelet Fc receptor. With MAIPA, GP-specific PA-IgG was demonstrated in half the patients with positive PA-IgG. Altogether, 20 to 30% of the patients with histories of thrombocytopenia were demonstrated to have GP-specific PA-IgG. Using MAIPA, Brighton et al (1996) found GP-specific PA-IgG in 49% (40/81) of patients with initially diagnosed immune thrombocytopenia and in 22% (11/51) of patients with non-immune thrombocytopenia, whereas Warner et al (1999), with MAIPA, found GP-specific PA-IgG in 39% (19/49) of patients with ITP and in 9% (3/32) of patients with initially diagnosed non-immune thrombocytopenia. The modification of MAIPA (Berchtold et al, 1997) with frozen platelet samples and monoclonal antibodies immobilising GP IIbIIIa and GP IbIX was found by

us to be suitable for the detection of GP-specific PA-IgG. The possibility that monoclonal antibody is directed against an epitope close to the specific target of the autoantibody could be ruled out by using different monoclonal antibodies against a single target protein but against different antigenic epitopes. Since GP-specific PA-IgG has also been detected in patients with various forms of disease-related thrombocytopenia (Berchtold et al, 1989c; Kurata et al, 1993; Pereira et al, 1995; Warner et al, 1999), GP-specific methods allow further subclassification and categorisation of autoimmune thrombocytopenic patients. A more thorough understanding of the significance of GP-specific PA-IgG is necessary.

Comparison of the PIFT with the direct MAIPA for the detection of PA-IgG showed that high fluorescence intensities for PA-IgG were associated with high ratios for GP-specific PA-IgG. Furthermore, it appeared that, in both studies, the thrombocytopenia in patients with strongly positive responses to both PA-IgG and GP-specific PA-IgG seemed possibly to be of autoimmune origin (Table 9). However, not all the positive PA-IgG could be attributed to GP IIbIIIa-, GP IbIX-, GP IaIIa- or GP V-specific antibodies, since almost half the patients with histories of thrombocytopenia remained negative in GP-specific MAIPA when the screening test was positive. Until the mechanisms of autoimmune thrombocytopenia have been fully clarified, it is also important to further characterise the specific PA-IgG in the different subpopulations of thrombocytopenic patients with increased peripheral platelet destruction due to different causes, and to further categorise the groups of patients according to their antibody profiles in addition to their clinical features. This approach should be widely evaluated and considered.

Little has been known about GP V-specific autoantibodies. In drug-induced thrombocytopenia, especially, GP V-specific PA-IgG may be present more frequently than has previously been suspected. GP V has rarely been demonstrated to be a target for platelet antibodies; GP V-specific circulating antibodies have been reported in two studies and a total of nine patients (Stricker & Shuman, 1986; Mayer & Beardsley, 1996). There have been no previous reports on the GP V specificity of PA-IgG.

When investigating GP specificity, the GP V-specific monoclonal antibody in a combination of different monoclonal antibodies should also be included. But with additional monoclonal antibodies one might find even more PA-IgG directed against other targets. It is to be hoped that definition of the characteristics of the platelet autoantibodies involved in autoimmune thrombocytopenia in terms of

glycoprotein target antigens and autoepitopes will help to explain the natural course and mechanism of platelet autoimmunity.

Circulating drug-dependent antibodies in the sera of patients with quinidine- or quinine-induced thrombocytopenia have mostly been reported to react with GP IIbIIIa or GP IbIX (Kunicki et al, 1978; van Leeuwen et al, 1982; Nieminen & Kekomäki, 1992; Burgess et al, 1998). Stricker & Shuman (1986) reported circulating GP V-specific quinidine-dependent antibodies in six patients with quinidine-induced thrombocytopenia. In the present study, one patient with quinidine-induced thrombocytopenia had no detectable GP IIbIIIa- or GP IbIX-specific PA-IgG, but strongly positive GP V-specific PA-IgG.

In gold-induced thrombocytopenia, patients with rheumatoid arthritis have been demonstrated to be strongly positive for PA-IgG (von dem Borne et al, 1986a; Adachi et al, 1987). However, GP-specific PA-IgG has not been reported. Here, the three patients with the strongest positivity for GP V-specific PA-IgG were on gold therapy and the additional case presented had definite gold-induced thrombocytopenia (Table 9). To the best of our knowledge, this is a new finding. The GP specificity of PA-IgG in gold-induced thrombocytopenia and in other forms of thrombocytopenia, especially drug-induced thrombocytopenia with a larger number of patients, calls for further investigation.

The material studied consisted of patient groups with heterogeneous histories of thrombocytopenia and also with other disorders. Characterisation of patients into well-defined clinically homogenous subgroups of autoimmune thrombocytopenia, especially in laboratory-based studies, seems difficult. A multicentre study, in tight collaboration with the laboratory facilities, would be needed to obtain homogenous, well-characterised groups of patients with exact criteria.

2. GENETIC POLYMORPHISM OF PLATELET FCγRIIA IN THROMBOCYTOPENIA

The polymorphism of FcγRIIA may have an effect on the pathogenesis of immune thrombocytopenia. In the groups of thrombocytopenic patients with different PA-IgG profiles, the distributions of FcγRIIA allele frequencies did not differ significantly from each other or from those of the controls. However, there was a tendency for RR131 clustering in patients with no detectable PA-IgG and also tendency for PA-IgG to be more abundant in patients with HH131 than in those with RR131. It is agreed that the H131R polymorphism of FcγRIIA affects the ligand binding properties of the receptor for the different IgG subclasses. Since the genetic polymorphism at residue 131 affects the functional properties of

FcγRIIA, Fcγ receptors may be responsible for the susceptibility to or modification of the disease. Several studies have aimed at clarifying the distribution of FcγRIIA allele frequencies in different groups of patients (Lehrnbecher et al, 1999). In light of the present studies, the evidence that disease susceptibility varies with FcγRIIA polymorphism remains weak (Table 5). Further studies of FcγRIIA alleles and probably other FcγR polymorphisms in specifically defined groups of thrombocytopenic patients would elucidate this question.

In patients with histories of thrombocytopenia and with heterogeneous backgrounds, the clustering of platelet FcγRIIA genotypes in the different groups according to age and gender is a new finding. Study III showed that patients with positive PA-IgG and no detectable GP-specific PA-IgG showed a tendency to have the genotype RR131. In the RR131 group, there were also more female patients under 40 years of age than in the HH131 group, suggesting different mechanisms of thrombocytopenia. Furthermore in the RR131 group, the male patients were older than the female patients. It is also known that among adults, ITP is more frequent in young women, 70% of female patients being under 40 years of age (George et al, 1994). Patients expressing H131 may have a different role for FcγRIIA in the clearance of PA-IgG, while individuals with R131 may have a different mechanism for developing autoimmune thrombocytopenia. Recently, patients with ITP were shown to have a tendency towards the RR131 genotype (Williams et al, 1998; Table 5).

Phagocytosis may be modulated by several mechanisms, which may include as yet unknown factors that influence the presentation of autoimmune thrombocytopenia. Platelet antibodies may trigger platelet activation by FcγRs and thus mediate more efficient clearance of platelets from the circulation. That platelets from females when stimulated by heat-aggregated IgG, released significantly more serotonin than platelets from males (Moore et al, 1981), has been suggested to imply either increased amounts of Fcγ receptors on platelets or more sensitivity to FcγR-induced platelet stimulation. Expression of FcγRs also varies on phagocytoses. Thus, the distribution of FcγRIIA allele frequencies should be studied not only in different immune-mediated disease conditions with well-defined characteristics, but also in heterogeneous groups of thrombocytopenic patients with known immunological properties. It seems likely that no single solution will be found, but rather the emphasis should be on the probable modifying effects of FcγRIIA and other FcγR polymorphisms.

3. MEASUREMENT OF RETICULATED PLATELETS IN THROMBOCYTOPENIA

The degree of fluorescence of thiazole orange (TO) stained platelets was analysed with methods based on two different principles. The results obtained with methods based on the intensity of the fluorescence (Patient median FL1) and the plot of FL versus FSC (% RP) showed no correlation, whereas those based on the relative intensity of the fluorescence (Relative FL1) and the plot of FL versus FSC (% RP) were correlated. An intra-assay control, a reference sample from a healthy blood donor, was applied to reduce the variation between the different test series. This same intra-assay control had a modifying effect on the results obtained with both Relative FL1 and % RP. This modifying effect may also have contributed to the positive correlation between Relative FL1 and % RP. In the measurements of reticulated platelets, it seems worthwhile to use a combination of different types of data analysis.

The proportion of reticulated platelets in normal adult controls has been shown to vary greatly, between 1% and 20% (Kienast & Schmitz, 1990; Bonan et al, 1993; Rinder et al, 1993; Richards & Baglin, 1995; Watanabe et al, 1995; Chavda et al, 1996), including inter-individual and inter-assay variation as well as the variation between the results obtained in different laboratories. Here, when the method based on plotted FL and FSC (% RP) was used, the level of TO positivity in normal samples was found to be between 4 and 6%. We did not aim at defining the exact percentages of RP. However, the range of median fluorescence intensity values (median FL1) for blood donor samples was rather wide, partly because it displayed the inter-assay variation and partly because it reflected the inter-individual variation.

Of the normal samples, the male controls were demonstrated to have higher fluorescence intensities for TO positivity than the female controls. Bonan et al (1993) also demonstrated a significant difference in the percentages of RP between normal males and females, the level being higher among the women. Such a sex-related difference has not been confirmed. From previous reports, a relation is known to exist between sex hormones and erythrocyte levels (Tarallo et al, 1994), but such a correlation has never been demonstrated for platelet levels, although oestrogen receptors have been identified in megakaryocytes (Tarantino et al, 1994). Thus, in the measurements of RP, careful selection of controls is important.

The binding of TO may be partly non-specific on accounting of the labelling of other platelet contents, such as dense granules (Robinson et al, 1998). The non-specific staining may be prevented by degranulating the platelets prior to the measurements. Like other colleagues (Ault et al, 1992; Dale et al, 1995; Richards et Baglin, 1995; Chavda et al, 1996; Joseph et al, 1996), we used RNAase to demonstrate

that the TO staining is RNA specific. The TO positivity was diminished in both patients and controls.

Yet, there are many different ways in which different laboratories use control samples, data analysis and interpretation of results. As controls, either lyophilised platelets, erythrocyte fluorescence, unstained samples, control cut-off values or RNAase treatment have been used (Ault et al, 1992; Bonan et al, 1993; Rinder et al, 1993; Romp et al, 1994; Richards & Baglin 1995; Saxon et al, 1998). Here, RNAase was not applied in every test series, rather an internal control sample was used. Standardisation of the procedures used in the analysis and investigations of RP and agreement in terms of sample preparation, labelling procedures, using of control samples, data analysis and interpretation of results would help to compare and combine the results obtained in different laboratories.

We were able to demonstrate a significant difference in TO positivity (Patient median FL1, Relative FL1) between pregnant thrombocytopenic women in the third trimester and healthy mothers with thrombocytopenic newborn infants, suggesting that the TO positivity could represent a real marker of an increased proportion of RP. The percentages of RP have been demonstrated to be significantly lower in pregnant women in the second trimester than in non-pregnant women (Rinder et al, 1994). In the first or third trimesters no difference could be seen between pregnant and non-pregnant women, in spite of a significant fall in the platelet count (Rinder et al, 1994). The fall in platelet count has been postulated to be due either to dilutional aetiology of thrombocytopenia or to down-regulation of thrombopoiesis in pregnancy. Significantly higher percentages of RP in four women with pre-eclampsia at 26 weeks, before the onset of any clinical signs, was suggested to represent a predictive value of RP in pregnant women at risk of hypertension and pre-eclampsia. This, together with our results, suggests that the measurement of RP may be clinically warranted.

SUMMARY AND CONCLUSIONS

Autoimmune thrombocytopenia is an immune-mediated bleeding disorder. Direct immunological methods for the detection of platelet antibodies have improved the specific measurements of platelet-associated antibodies (PA-IgG). Although the findings of PA-IgG and also glycoprotein (GP)-specific PA-IgG methods give additional knowledge of the disorder, understanding of the immunopathological mechanisms and the presence of platelet antibodies in different conditions is still incomplete.

The aims of this study were to investigate the presence of PA-IgG and also GP-specific PA-IgG in patients with thrombocytopenia. The possible target role of GP V was investigated separately (I, II). Further, we aimed to elucidate the distribution of the platelet Fcγ receptor (FcγR) polymorphism in thrombocytopenic patients with different PA-IgG profiles, i.e. the possible association between FcγRIIA polymorphism H131R and PA-IgG (III). We also evaluated the measurement of reticulated platelets (RP), presumed markers of thrombopoiesis, by using thiazole orange staining and flow cytometry (IV). Our material comprised patients with heterogeneous histories of thrombocytopenia and also with other disorders. Most of our patients could be divided into groups of, for example, ITP, drug-induced thrombocytopenia, pregnancy-associated thrombocytopenia and disease-related thrombocytopenia, although a large proportion of the patients could not be placed in any of these categories (I-III). Study IV comprised a total of 50 samples from pregnant women with thrombocytopenia, thrombocytopenic women after full-term pregnancy and healthy women with a thrombocytopenic newborn infant.

The results of the PA-IgG screening test, i.e. the direct PIFT, and direct MAIPA, for detection of GP-specific PA-IgG, could be used to subclassify the patients further. A total of 159 and also of 882 patients with heterogeneous histories of thrombocytopenia were screened for PA-IgG, and half the patients were found to be positive for PA-IgG (I, II). Furthermore, GP IIbIIIa- and/or IbIX- and/or IaIIa-specific PA-IgG was present in approximately half the patients with positive PA-IgG. The patients with strongly positive PA-IgG, detected both with PIFT and with MAIPA, seemed to be clinically more homogeneous and to have thrombocytopenia with autoimmune features. Thus, the importance of GP IIbIIIa and GP IbIX as target antigens of PA-IgG was confirmed. Interestingly, in further studies, GP V was found to be an important target antigen. One patient with quinidine-induced thrombocytopenia had no detectable GP IIbIIIa- or GP IbIX-specific PA-IgG, but was strongly positive for GP V-specific PA-IgG. GP V-specific PA-IgG was found in five patients with thrombocytopenia due to gold therapy. These are new findings.

We hoped that categorisation of patients with heterogeneous forms of thrombocytopenia according to FcγRIIA genotypes would elucidate the multifactorial mechanisms of thrombocytopenia. The clustering of FcγRIIA genotypes in the different groups of patients, a total of 113 patients, according to age and gender was a new finding. The distribution of the platelet FcγRIIA polymorphism was also determined in 93 Finnish blood donors; the allele distribution did not differ significantly from that of other Caucasians. The laboratory methods used for the genotyping of platelet FcγRIIA polymorphism and for the detection of platelet antibodies may be considered reliable and reproducible. However, the significance of the polymorphism in the disease susceptibility still remains unsolved. Prospective studies of clinically selected groups of patients should help to clarify whether there is any connection between FcγRIIA polymorphism and the immunological mechanisms, especially in ITP.

In the measurements of reticulated platelets (a total of 50 patients), significant differences were observed between pregnant thrombocytopenic women and healthy women in the fluorescence of thiazole orange-stained platelets. Furthermore, a significant difference was also noticed in PA-IgG profiles; pregnant thrombocytopenic women had higher levels of PA-IgG than healthy women. Patients with positive PA-IgG also tended to have high fluorescence intensities for RP. There is as yet no standardisation of the procedures used in the analysis of RP, but our present study confirms that measurement of RP provides an additional non-invasive means of evaluating the pathogenetic mechanisms of thrombocytopenia. Internationally agreed consensus about the laboratory methodology would accelerate research in this field.

Subclassification of patients into different categories according to laboratory findings seems worth while as long as autoimmune thrombocytopenia remains a group of heterogeneous disorders. A large, perhaps multinational study, would be needed to collect groups of thrombocytopenic patients with clinical characteristics sufficiently homogeneous for further subclassification to establish positive diagnostic criteria.

ACKNOWLEDGEMENTS

This work was carried out at the Finnish Red Cross Blood Transfusion Service in Helsinki. I wish to express my sincere gratitude to the Head of the Blood Transfusion Service, Professor Juhani Leikola, M.D., for placing such excellent research facilities at my disposal.

I warmly thank my supervisor, Docent Riitta Kekomäki, M.D., for introducing me to the interesting and fascinating world of science. Her guidance and deep expertise in platelet serology were crucial for this work. With knowledge and broad vision, she has promoted platelet immunology in Finland to a high standard. Her precision, persistence and interest in every aspect are beyond comparison.

I am indebted to all my collaborators at the Blood Transfusion Service and at the clinic. I warmly thank Kaija Javela, M.Sc., and Docent Jukka Partanen, Ph.D., for their skilful help, knowledge and encouragement. Pirkko Hormila, M.D., Docent Seija Riikonen, M.D., Susanna Sainio, M.D., and Professor Kari Teramo, M.D., are acknowledged for their clinical expertise and pleasant collaboration.

Docent Erkki Elonen, M.D., and Docent Eeva-Riitta Savolainen, M.D., are warmly thanked for carefully reviewing the thesis and for their constructive comments.

My special thanks go to Professor Eero Ikkala, M.D., and Professor Gunnar Myllylä, M.D., for their pioneer work in the field of clinical and laboratory haematology. Docent Tom Krusius, M.D., is thanked for being a rational, supportive and understanding boss. The help of other members of the senior scientific community at the Blood Transfusion Service is also acknowledged: I am especially grateful to Docent Anne Mäkipernaa, M.D., for her cheerful encouragement and interest in my work and Docent Vesa Rasi, M.D., for teaching me the secrets of the coagulation system.

The skilful help of the staff of the Platelet Laboratory, Anu, Iris, Kirsi, Outi, Pirjo, Päivi, Reetta, Tuija and Ulla is acknowledged. I appreciate their friendly attitude towards my sometimes hectic schedules.

I wish to address my special thanks to Mrs Jean Margaret Perttunen, B.Sc. (Hons), for careful revision of the language of my thesis. Marja-Leena Hyvönen, M.A. and Ms. Maija Ekholm are thanked for the excellent library services.

I was privileged to have such great workmates. The friendliness of junior scientific community of the Blood Transfusion Service is acknowledged. Satu Koskela is warmly thanked for her sincere friendship and for sharing many valuable thoughts and sometimes even emotional moments. Satu Kekomäki is thanked for our unforgettable trips and the cheerful times in the lab. Leena Hakala is thanked for the enjoyable chats and her loyal, warm friendship. The other members of the "Underground Network" are also thanked: Kristiina Kuismanen for her friendliness and Malla Kuosmanen for her cheerful, sympathetic attitude. I warmly thank Taina Jaatinen and the other "Tissue-typers" Päivi Holopainen, Kati Karell, Nina Klinger, Antti Levo, Anne Polvi and Kaisa Poutanen as well as Pekka Aroviita, Leena Capraro, Päivi Helminen and Laura Kerosuo for making these years enjoyable and memorable. I also want to thank my "charismatic" friends at the Medical School, "the Flickorna" Piia Aarnisalo, Mari Hero, Satu Kalliola and also Sanna Toppila, with whom I have shared great times during these years.

The financial support from the Blood Disease Research Foundation in Finland, the Finnish Association of Haematology, the Finnish Cultural Foundation in Satakunta, the Finnish Medical Foundation and the Foundation for Promoting Laboratory Medicine is gratefully acknowledged.

I owe my deepest and warmest gratitude to my parents, Leena and Jaakko, for their never-failing encouragement and love. I appreciate their continuous support - also financial - and sincere interest in my work and in my life. My brother Teemu is thanked for introducing me to the world of medicine and for his rational opinions and answers to everything.

My warmest thanks go to my husband Timo for his patience, support, help and love. Without Timo's encouragement this work would have been much more difficult to complete.

Helsinki, May 2000

REFERENCES

Adachi JD, Bensen WG, Kassam Y, Powers PJ, Bianchi FA, Cividino A, Kean WF, Rooney PJ, Craig GL, Buchanan WW, Tugwell PX, Gordon DA, Lucarelli A, Singal DP. Gold induced thrombocytopenia: 12 cases and a review of the literature. Semin Arthritis Rheum 1987;16:287-93.

Aliberti G, Proietta M, Pulignano I, de Franceschi L. Differences in platelet count and size between marrow and peripheral blood. Haemostasis 1996;26:276-83.

Amiral J, Bridey F, Wolf M, Boyer-Neumann C, Fressinaud E, Vissac AM, Peynaud-Debayle E, Dreyfus M, Meyer D. Antibodies to macromolecular platelet factor 4-heparin complexes in heparin-induced thrombocytopenia: a study of 44 cases. Thromb Haemost 1995;73:21-8.

Anderson CL, Ryan DH, Looney RJ, Leary PC. Structural polymorphism of the human monocyte 40 kilodalton Fc receptor for IgG. J Immunol 1987;138:2254-6.

Anderson GP, van de Winkel JGJ, Anderson CL. Anti-GPIIb/IIIa (CD41) monoclonal antibody-induced platelet activation requires Fc receptor-dependent cell-cell interaction. Br J Haematol 1991;79:75-83.

Anderson CL, Chacko GW, Osborne JM, Brandt JT. The Fc receptor for immunoglobulin G (FcγRII) on human platelets. Semin Thromb Hemost 1995;21:1-9.

Arepally G, McKenzie SE, Jiang X-M, Poncz M, Cines DB. FcγRIIA H/R^{131} polymorphism, subclass-specific IgG anti-heparin/platelet factor 4 antibodies and clinical course in patients with heparin-induced thrombocytopenia and thrombosis. Blood 1997;89:370-5.

Aster RH, Jandl JH. Platelet sequestration in man. II. Immunological and clinical studies. J Clin Invest 1964;43:856-69.

Aster RH. Drug-induced immune thrombocytopenia: An overview of pathogenesis. Semin Hematol 1999;36:2-6.

Atsumi T, Caliz R, Amengual O, Khamashta MA, Hughes GRV. Fcγ receptor IIA H/R131 polymorphism in patients with antiphospholipid antibodies. Thromb Haemost 1998;79:924-7.

Ault KA. Flow cytometric measurement of platelet-associated immunoglobulin. Pathology and Immunopathology Research 1988;7:395-408.

Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CPH, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. Am J Clin Pathol 1992;98:637-46.

Ault KA. Flow cytometric measurement of platelet function and reticulated platelets. Ann NY Acad Sci 1993;677:293-308.

Ault KA, Knowles C. In vivo biotinylation demonstrates that reticulated platelets are the youngest platelets in circulation. Exp Hematol 1995;23:996-1001.

Bachelot C, Saffroy R, Gandrille S, Aiach M, Rendu F. Role of FcγRIIA gene polymorphism in human platelet activation by monoclonal antibodies. Thromb Haemost 1995;74:1557-63.

Bachelot-Loza C, Saffroy R, lasne D, Chatellier G, Aiach M, Rendu F. Importance of the FcγRIIa-Arg/His-131 polymorphism in heparin-induced thrombocytopenia diagnosis. Thromb Haemost 1998;79:523-8.

Ballem PJ, SegalaGM, Stratton JR, Gernsheimer T, Adamson JW, Slichter SJ. Mechanisms of thrombocytopenic in chronic autoimmune thrombocytopenic purpura. Evidence of both impaired platelet production and increased platelet clearance. J Clin Invest 1987;80:33-40.

Beardsley DS, Spigel JE, Jacobs MM, Handin RI, Lux SE. Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J Clin Invest 1984;74:1701-7.

Beer JH, Rabaglio M, Berchtold P, von Felten A, Clemetson KJ, Tsakiris DA, Kehrel B,
Brandenberger. Autoantibodies against the platelet glycoproteins (GP) IIb/IIIa, Ia/IIa, and IV and particular deficiency in GPIV in a patient with bleeding disorder and a defective platelet collagen interaction. Blood 1993;82:820-9.

Beer JH, Buchi L, Steiner B. Glycocalicin: a new assay - the normal plasma levels and its potential usefulness in selected diseases. Blood 1994;83:691-702.

Berchtold P, McMillan R, Tani P, Sommerville-Nielsen S, Blanchette VS. Autoantibodies against platelet membrane glycoproteins in children with acute and chronic immune thrombocytopenia. Blood 1989a;74:1600-2.

Berchtold P, McMillan R. Therapy of chronic idiopathic thrombocytopenic purpura in adults. Blood 1989b; 74:2309-17.

Berchtold P, Harris JP, Tani P, Piro L, McMillan R. Autoantibodies to platelet glycoproteins in patients with disease-related immune thrombocytopenia. Br J Haematol 1989c;73:365-8.

Berchtold P, Wenger M. Autoantibodies against platelet glycoproteins in autoimmune thrombocytopenic purpura: their clinical significance and response to treatment. Blood 1993;81:1246-50.

Berchtold P, Muller D, Beardsley D, Fujisawa K, Kaplan C, Kekomäki R, Lipp E, Morell-Kopp MC, Kiefel V, McMillan R, von dem Borne AEGKr, Imbach P. International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies. Br J Haematol 1997;96:477-83.

Berkman N, Michaeli Y, Or R, Eldor A. EDTA-dependent pseudothrombocytopenia: a clinical study of 18 patients and a review of the literature. Am J Hematol 1991;36:195-201.

Berndt MC, Mazurov AV, Vinogradov DV, Burns GF, Chesterman CN. Topographical association of the platelet Fc-receptor with the glycoprotein IIb-IIIa complex. Platelets 1993;4:190-6.

Bettaieb A, Fromont P, Louache F, Oksenhendler E, Vainchenker W, Duedari N, Bierling P. Presence of cross-reactive antibody between human immunodeficiency virus (HIV) and platelet glycoproteins in HIV-related immune thrombocytopenic purpura. Blood 1992;80:162-9.

Bierling P, Bettaieb A, Fromont P, Favrin M, Duedari N. Anti-GMP140 (CD 62) autoantibody in a patient with autoimmune thrombocytopenic purpura. Br J Haematol 1994;87:631-3.

Bolton FG. Thrombocytopenic purpura due to quinidine. II. Serologic mechanisms. Blood 1956;11:547-64.

Bonan JL, Rinder HM, Smith BR. Determination of the percentage of thiazole orange (TO)-positive, "reticulated" platelets using autologous erythrocyte TO fluorescence as an internal standard. Cytometry 1993;14:690-4.

Boneu B, Corberand J, Plante J, Bierme R. Evidence that platelet density and volume are not related to aging. Thromb Res 1977;10:475-86.

von dem Borne AEGKr, Verheugt FWA, Oosterhof F, von Riesz E, Brutel de la Riviere A, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol 1978;39:195-207.

von dem Borne AEGKR, Helmerhorst FM, van Leeuwen EF, Pegels HG, von Riesz E, Engelfriet CP. Autoimmune thrombocytopenia: detection of platelet autoantibodies with the suspension immunofluorescence test. Br J Haematol 1980;45:319-27.

von dem Borne AEGKr, Pegels JG, van der Stadt RJ, van der Plas-van Dalen CM, Helmerhorst FM. Thrombocytopenia associated with gold therapy: a drug-induced autoimmune disease? Br J Haematol 1986a;63:509-16.

von dem Borne AEGKr, Vos JJE, van der Lelie J, Bossers B, van Dalen CM. Clinical significance of positive platelet immunofluorescence test in thrombocytopenia. Br J Haematol 1986b;64:767-76.

Botto M, Theodoridis E, Thompson EM, Beynon HLC, Briggs D, Isenberg DA, Walport MJ, Davies KA. FcγRIIa polymorphism in systemic lupus erythematosus (SLE): no association with disease. Clin Exp Immunol 1996;104:264-8.

Bowditch RD, Tani P, Fong KC, McMillan R. Characterization of autoantigenic epitopes on platelet glycoprotein IIb/IIIa using random peptide libraries. Blood 1996;88:4579-84.

Brandt JT, Isenhart CE, Osborne JM, Ahmed A, Anderson CL. On the role of platelet FcγRIIa phenotype in heparin-induced thrombocytopenia. Thromb Haemost 1995;74:1564-72.

Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood 1996;88:194-201.

Burgess JK, Lindeman R, Chesterman CN, Chong BH. Single amino acid mutation of Fcγ receptor is associated with the development of heparin-induced thrombocytopenia. Br J Haematol 1995;91:761-6.

Burgess JK, Lopez JA, Berndt MC, Dawes I, Chesterman CN, Chong BH. Quinine-dependent antibodies bind a restricted set of epitopes on the glycoprotein Ib-IX complex: characterization of the epitopes. Blood 1998;92:2366-73.

Burrows RF, Kelton JG. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. N Engl J Med 1993;329:1463-6.

Carlsson LE, Santoso S, Baurichter G, Kroll H, Papenberg S, Eichler P, Westerdaal NAC, Kiefel V, van de Winkel JGJ, Greinacher A. Heparin-induced thrombocytopenia: new insights into the impact of the FcγRIIa-R-H131 polymorphism. Blood 1998;92:1526-31.

Cassel DL, Keller MA, Surrey S, Schwartz E, Schreiber AD, Rappaport EF, McKenzie SE. Differential expression of FcγRIIA, FcγRIIB and FcγRIIC in hematopoietic cells: analysis of transcripts. Mol Immunol 1993;30:451-60.

Chavda N, Mackie IJ, Porter JB, Harrison P, Patterson K, Machin SJ. Rapid flow cytometric quantitation of reticulated platelets in whole blood. Platelets 1996;7:189-94.

Chong BH, Pilgrim R, Cooley MA, Chesterman CN. Increased expression of platelet IgG Fc receptors in immune heparin-induced thrombocytopenia. Blood 1993;81:988-93.

Christie DJ, Mullen PC, Aster RH. Quinine- and quinidine platelet antibodies can react with GPIIb/IIIa. Br J

Haematol 1987;67:213-9.

Christopoulos CG, Kelsey HC, Machin SJ. A flow-cytometric approach to quantitative estimation of platelet surface immunoglobulin G. Vox Sang 1993;64:106-15.

Clark MR, Clarkson SB, Ory PA, Stollman N, Goldstein IM. Molecular basis for a polymorphism involving Fc receptor II on human monocytes. J Immunol 1989;143:1731-4.

Clark MR, Stuart SG, Kimberly RP, Ory PA, Goldstein IM. A single amino acid distinguishes the high-responder from the low-responder form of Fc receptor II on human monocytes. Eur J Immunol 1991;21:1911-6.

Corash L, Tan H, Gralnick HR. Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure. Blood 1977;49:71-87.

Cordiano I, Salvan F, Randi ML, Ruffatti MA, Steffan A, Girolami A, Fabris F. Antiplatelet glycoprotein autoantibodies in patients with autoimmune diseases with and without thrombocytopenia. J Clin Immunol 1996;16:340-6.

Cordiano I, Betterle C, Spadaccino CA, Soini B, Girolami A, Fabris F. Autoimmune thrombocytopenia (AITP) and thyroid autoimmune disease (TAD): overlapping syndromes?. Clin Exp Immunol 1998;113:373-8.

Court WS, Bozeman JM, Soong Sj, Saleh MN, Shaw DR, LoBuglio AF. Platelet surface-bound IgG in patients with immune and nonimmune thrombocytopenia. Blood 1987;69:278-83.

Crossley A, Calvert JE, Taylor PRA, Dickinson AM. A comparison of monoclonal antibody immobilization of platelet antigen (MAIPA) and immunobead methods for detection of GPIIb/IIIa antiplatelet antibodies in immune thrombocytopenic purpura. Transfus Med 1997;7:127-34.

Dale GL, Friese P, Hynes LA, Burstein SA. Demonstration that thiazole-orange -positive platelets in the dog are less that 24 hours old. Blood 1995;85:1822-5.

Dale GL. Platelet kinetics. Curr Opin Hematol 1997;4:330-4.

Deckmyn H, Chew SL, Vermylen J. Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. Thromb Haemost 1990;64:74-9.

Deckmyn H, Zhang J, van Houtte E, Vermylen J. Production and nucleotide sequence of an inhibitory human IgM autoantibody directed against platelet glycoprotein Ia/IIa. Blood 1994;84:1968-74.

Deckmyn H, Vanhoorelbeke K, Peerlinck K. Inhibitory and activating human antiplatelet antibodies. Baillieres Clin Haematol 1998;11:343-59.

Denomme GA, Warkentin TE, Horsewood P, Sheppard J-AI, Warner MN, Kelton JG. Activation of platelets by sera containing IgG1 heparin-dependent antibodies: an explanation for the predominance of the FcγRIIa “low responder” (his131) gene in patients with heparin-induced thrombocytopenia. J Lab Clin Med 1997;130:278-84.

Diehl LF, Ketchum LH. Autoimmune disease and chronic lymphocytic leukemia: autoimmune hemolytic anemia, pure red cell aplasia, and autoimmune thrombocytopenia. Semin Oncol 1998;25:80-97.

Dixon R, Rosse W, Ebbert L. Quantitative determination of antibody in idiopathic thrombocytopenic purpura. Correlation of serum and platelet-bound antibody with clinical response. N Engl J Med 1975;230-6.

Doan CA, Bouroncle BA, Wiseman BK. Idiopathic and secondary thrombocytopenic purpura: clinical study and

evaluation of 381 cases over a period of 28 years. Ann Intern Med 1960;53:861-76.

Duits AJ, Bootsma H, Derksen RHWM, Spronk PE, Kater L, Kallenberg CGM, Capel PJA, Westerdaal NAC, Spierenburg GTH, Gmeglig-Meyling FHJ, van de Winkel JGJ. Skewed distribution of IgG Fc receptor IIa (CD32) polymorphism is associated with renal disease in systemic lupus erythematosus patients. Arthritis Rheum 1995;39:1832-6.

Emmons RVB, Reid DM, Cohen RL, Meng G, Young NS; Dunbar CE, Shulman NR. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood 1996;87:4068-71.

Fabris F, Steffan A, Cordiano I, Borzini P, Luzzatto G, Randi ML, Girolami A. Specific antiplatelet autoantibodies in patients with antiphospholipid antibodies and thrombocytopenia. Eur J Haematol 1994;53:232-6.

Flesch BK, Bauer F, Neppert J. Rapid typing of the human Fcγ receptor IIA polymorphism by polymerase chain reaction amplification with allele-specific primers. Transfusion 1998;38:174-6.

Fujisawa K, O´Toole TE, Tani P, Loftus JC, Ginsberg MH, McMillan R. Autoantibodies to the presumptive cytoplasmic domain of platelet glycoprotein IIIa in patients with chronic immune thrombocytopenia. Blood 1991;77:2207-13.

Fujisawa K, Tani P, O´Toole TE, Ginsberg MH, McMillan R. Different specificities of platelet-associated and plasma autoantibodies to platelet GP IIb-IIIa in patients with chronic immune thrombocytopenic purpura. Blood 1992;79:1441-6.

Fujisawa K, Tani P, McMillan R. Platelet-associated antibody to glycoprotein IIb/IIIa from chronic immune thrombocytopenic purpura patients often binds to divalent cation-dependent antigens. Blood 1993;81:1284-9.

Gaiger A, Neumeister A, Heinzl H, Pabinger I, Panzer S. HLA class-I and -II antigens in chronic idiopathic autoimmune thrombocytopenia. Ann Hematol 1994;68:299-302.

Galli M, Daldossi M, Barbui T. Anti-glycoprotein Ib/IX and IIb/IIIa antibodies in patients with antiphospholipid antibodies. Thromb Haemost 1994;71:571-5.

George JN. Platelet IgG: measurement, interpretation, and clinical significance. Prog Hemost Thromb 1991:10;97-126.

George JN, El-Harake MA, Raskob GE. Chronic idiopathic thrombocytopenic purpura. N Engl J Med 1994;331:1207-11.

George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, Ballem PJ, Blanchette VS, Bussel JB, Cines DB, Kelton JG, Lichtin AE, McMillan R, Okerbloom JA, Regan DH, Warrier I. Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. Blood 1996;88:3-40.

Godeau B, Piette J-C, Fromont P, Intrator L, Schaeffer A, Bierling P. Specific antiplatelet glycoprotein autoantibodies are associated with the thrombocytopenia of primary antiphospholipid syndrome. Br J Haematol 1997;98:873-9.

Gonzalez-Conejero R, Rivera J, Rosillo MC, Cano A, Rodriguez T, Vicente V. Association of autoantibodies against platelet glycoproteins Ib/IX and IIb/IIIa, and platelet-reactive anti-HIV antibodies in thrombocytopenic narcotic addicts. Br J Haematol 1996;464-71.

Gratama JW, D´amaro J, de Koning J, den Ottolander GJ. The HLA-system in immune thrombocytopenic purpura:

its relation to the outcome of therapy. Br J Haematol 1984;56:287-93.

Greinacher A, Pötzsch, Amiral J, Dummel V, Eicher A, Mueller-Eckhardt C. Heparin-associated thrombocytopenia: isolation of the characterization of a multimolecular PF4-heparin complex as the major antigen. Thromb Haemost 1994a;71:247-51.

Greinacher A, Amiral J, Dummel V, Vissac A, Kiefel V, Mueller-Eckhardt C. Laboratory diagnosis of heparin-associated thrombocytopenia and comparison of platelet aggregation test, heparin-induced platelet activation test, and platelet factor 4/heparin enzyme-linked immunosorbent assay. Transfusion 1994b;34:381-5.

de Haas M, Kleijer M, van Zwieten R, Roos D, von dem Borne AEGKr. Neutrophil FcγRIIIb deficiency, nature, and clinical consequences: a study of 21 individuals from 14 families. Blood 1995;86:2403-13.

Harrington WJ, Minnich V, Hollinsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J Lab Clin Med 1951;38:1-10.

Hasegawa Y, Nagasawa T, Kamoshita M., Komeno T, Itoh T, Ninomiya H, Abe T. Effects of anti-platelet glycoprotein Ib and/or IIb/IIIa autoantibodies on the size of megakaryocytes in patients with immune thrombocytopenia. Eur J Haematol 1995;55:152-7.

He R, Read DM, Jones CE, Shulman NR. Spectrum of Ig classes, specificities, and titers of serum antiglycoproteins in chronic idiopathic thrombocytopenic purpura. Blood 1994;83:1024-32.

He R, Reid DM, Jones CE, Shulman NR. Extracellular epitopes of platelet glycoproteins Ibα reactive with serum antibodies from patients with chronic idiopathic thrombocytopenic purpura. Blood 1995;86:3789-6.

Hebbar M, Kaplan C, Caulier MT, Wattel E, Morel E, Wetterwald M, Bauters F, Fenaux P. Low incidence of specific anti-platelet antibodies detected by the MAIPA assay in the serum of thrombocytopenic MDS patients and lack of correlation between platelet autoantibodies, platelet lifespan and response to danazol therapy. Br J Haematol 1996;94:112-5.

Helmerhorst FM, Bossers B, de Bruin HG, Engelfriet CP, von dem Borne AEGKr. Detection of platelet antibodies: a comparison of three techniques. Vox Sang 1980;39:83-92.

Helmerhorst FM, Nijenhuis LE, de Lange GG, van den Berg-Loonen PM, Jansen MFM, von dem Borne AEGKr, Engelfriet CP. HLA antigens in idiopathic thrombocytopenic purpura. Tissue Antigens 1982a;20:372-9.

Helmerhorst FM, van Leeuwen EF, Pegels JG, van der Plas-van Dalen C, Eengelfriet CP, von dem Borne AEGKr. Primary and secondary autoimmune thrombocytopenia. A serological and clinical analysis. Scand J Haematol 1982b;28:319-28.

Hou M, Stockelberg D, Kutti J, Wadenvik H. Naturally occurring IgG autoantibodies to platelet cytoskeleton tropomyosin. Thromb Haemost 1996;75:642-7.

Hulett MD, Witort E, Brinkworth RI, Mckenzie IFC, Hogarth PM. Identification of the IgG binding site of the human low affinity receptor for IgG FcγRII. J Biol Chem 1994;269:15287-93.

Hürlimann-Forster M, Steiner B, von Felten A. Quantitation of platelet-specific autoantibodies in platelet eluates of ITP patients measured by a novel ELISA using the purified glycoprotein complexes GPIIb/IIIa and GPIb/IX as antigens. Br J Haematol 1997;98:328-35.

Ichikawa N, Ishida F, Shimodaira S, Tahara T, Kato T, Kitano K. Regulation of serum thrombopoietin levels by platelets and megakaryocytes in patients with aplastic anaemia and idiopathic thrombocytopenic purpura. Thromb

Haemost 1996;76:156-60.

Imbach P, Tani P, Berchtold W, Blanchette V, Burek-Kozlowska A, Gerber H, Jacobs P, Newland A, Turner C, Wood L, McMillan R. Different forms of chronic childhood thrombocytopenic purpura defined by antiplatelet autoantibodies. J Pediatr 1991;118:535-9.

Ingram M, Coopersmith A. Reticulated platelets following acute blood loss. Br J Haematol 1969;17:225-9.

International Committee for Standardization in Hematology Panel on Diagnostic Applications of Radionuclides. Recommended method for Indium-111 platelet survival studies. J Nucl Med 1988;29:564-6.

Jackson SR, Carter JM. Platelet volume: Laboratory measurement and clinical application. Blood Reviews 1993;7:104-13.

Jiang X-M, Arepally G, Poncz M, McKenzie SE. Rapid detection of the FcγRIIA-H/R131 ligand-binding polymorphism using an allele-specific restriction enzyme digestion (ASRED). J Immunol Methods 1996;199:55-9.

Jouhikainen T, Kekomäki R, Leirisalo-Repo M, Bäcklund T, Myllylä G. Platelet autoantibodies detected by immunoblotting in systemic lupus erythematosus: association with the lupus anticoagulant, and with history of thrombosis and thrombocytopenia. Eur J Haematol 1990;44:234-9.

Joseph MA, Adams D, Maragos J, Saving KL. Flow cytometry of neonatal platelet RNA. J Pediatr Hematol Oncol 1996;18:277-81.

Jy W, Horstman LL, Arce M, Ahn YS. Clinical significance of platelet microparticles in autoimmune thrombocytopenias. J Lab Clin Med 1992;119:334-45.

Kaplan C, Morinet F, Cartron J. Virus-induced autoimmune thrombocytopenia and neutropenia. Semin Hematol 1992;29:34-44.

Karas SP, Rosse WF, Kurlander RJ. Characterization of the IgG-Fc receptor on human platelets. Blood 1982;60:1277-82.

Kaushansky K. Thrombopoietin. N Engl J Med 1998;339:746-54.

Kekomäki R, Myllylä G. Effect of normal human serum on the binding of specific antibodies and platelet-unrelated immune complexes to human platelets. Scand J Immunol 1979;9:527-35.

Kekomäki R, Rajamäki A, Myllylä G. Detection of quinidine-specific antibodies with platelet 125I-labeled staphylococcal protein A test. Vox Sang 1980;38:12-18.

Kekomäki R, Dawson B, McFarland J, Kunicki TJ. Localization of human platelet autoantigens to the cysteine-rich region of GP IIIa. J Clin Invest 1991;88:847-54.

Kelton JG, Meltzer D, Moore J, Giles AR, Wilson WE, Barr R, Hirsh J, Neame PB, Powers PJ, Walker I, Bianchi F, Carter CJ. Drug-induced thrombocytopenia is associated with increased binding of IgG to platelets both in vivo and in vitro. Blood 1981;58: 524-9.

Kelton JG, Powers PJ, Carter CJ. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. Blood 1982;60:1050-3.

Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood 1987;70:1722-6.

Kiefel V, Santoso S, Kaufmann E, Mueller-Eckhardt C. Autoantibodies against platelet glycoprotein Ib/IX: a frequent finding in autoimmune thrombocytopenic purpura. Br J Haematol 1991;79:256-62.

Kiefel V. The MAIPA assay and its applications in immunohaematology. Transfus Med 1992;2:181-8.

Kiefel V, Freitag E, Kroll H, Santoso S, Mueller-Eckhardt C. Platelet autoantibodies (IgG, IgM, IgA) against glycoproteins IIb/IIIa and Ib/IX in patients with thrombocytopenia. Ann Hematol 1996;72:280-5.

Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood 1990;75:116-21.

Kimberley RP, Salmon JE, Edberg JC. Receptors for immunoglobulin G. Arthritis Rheum 1995;38:306-14.

Klimiuk PS, Holt PJL, Clague RB, Herod E, Dyer PA. Predictive value of mean platelet volume in gold induced thrombocytopenia. Ann Rheum Dis 1987;46:38-41.

Kobayashi T, Westerdaal NAC, Miyazaki A, van der Pol WL, Suzuki T, Yoshie H, van de Winkel JGJ, Hara K. Relevance of immunoglobulin G Fc receptor polymorphism to recurrence of adult periodontitis in Japanese patients. Infect Immun 1997;65:3556-60.

Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AEGKr, de Haas M. FcγRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell FcγRIII1, independently of the FcγRIIIa-48L/R/H phenotype. Blood 1997;90:1109-14.

Koike Y, Yoneyama, Shirai J, Ishida T, Shoda E, Miyaki K, Sunaga S, Horie R, Aoki K, Koike K, Ogata I, Tahara T, Kato T, Nakahara K, Katiya T, Higashihara M. Evaluation of thrombopoiesis in thrombocytopenic disorders by simultaneous measurement of reticulated platelets of whole blood and serum thrombopoietin concentrations. Thromb Haemost 1998;79:1106-10.

Kokawa T, Nomura S, Yasunaga K. Relationship between platelet volume and anti-platelet autoantibodies in idiopathic thrombocytopenic purpura. Eur J Haematol 1991;47:104-8.

Kokawa T, Nomura S, Yanabu M, Yasunaga K. Detection of platelet antigen for antiplatelet antibodies in idiopathic thrombocytopenic purpura by flow cytometry, antigen-capture ELISA, and immunoblotting: a comparative study. Eur J Haematol 1993;50:74-80.

Koskela S. Genetics of the platelet glycoprotein Ib/IX/V complex in families with Bernard-Soulier syndrome and in the normal population of Finland. Academic dissertation, University of Helsinki, 1999.

Kosty MP, Hench PK, Tani P, McMillan R. Thrombocytopenia associated with auranofin therapy: evidence for a gold-dependent immunologic mechanism. Am J Hematol 1989;30:236-9.

Kosugi S, Kurata Y, Tomiyama Y, Tahara T, Kato T, Tadokoro S, Shiraga M, Honda S, Kanakura Y, Matsuzawa Y. Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. Br J Haematol 1996a;93:704-6.

Kosugi S, Tomiyama Y, Shiraga M, Kashiwagi H, Mizutani H, Kanakura Y, Kurata Y, Matsuzawa. Platelet-associated anti-glycoprotein IIb-IIIa autoantibodies in chronic immune thrombocytopenic purpura mainly recognize cation-dependent conformations: comparison with the epitopes of serum autoantibodies. Thromb Haemost 1996b;75:339-45.

Kunicki TJ, Johnson MM, Aster RH. Absence of the platelet receptor for drug-dependent antibodies in the Bernard-Soulier syndrome. J Clin Invest 1978;62:716-9.

Kunicki TJ, Furihata K, Kekomäki R, Scott JP, Nugent DJ. A human monoclonal antibody specific for human platelet glycoprotein IIb (integrin IIb) heavy chain. Hum Antib Hyb 1990;1:83-95.

Kunicki TJ, Plow EF, Kekomäki R, Nugent DJ. Human monoclonal autoantibody 2E7 is specific for a peptide sequence of platelet GP IIb. Localization of the epitope to IIb231-238 with an immunodominant Trp235. J Autoimmunity 1991a;4:415-31.

Kunicki TJ, Annis DS, Gorski J, Nugent DJ. Nucleotide sequence of the human autoantibody 2E7 specific for the platelet integrin IIb heavy chain. J Autoimmun 1991b;4:433-46.

Kunicki TJ, Newman PJ. The molecular immunology of human platelet proteins. Blood 1992;80:1386-404.

Kurata Y, Hayashi S, Kosugi S, Kashiwagi H, Tomiyama Y, Kanayama Y, Matsuzawa Y. Elevated platelet-associated IgG in SLE patients due to anti-platelet autoantibody: differentiation between autoantibodies and immune complexes by ether elution. Br J Haematol 1993;85:723-8.

Lanza F, Kieffer N, Phillips DR, Fitzgerald LA. Characterization of the human platelet glycoprotein IIIa gene. Comparison with the fibronectin receptor beta-subunit gene. J Biol Chem 1990;265:20801-7.

Lee LG, Chen C-H, Chiu LA. Thiazole orange: a new dye for reticulocyte analysis. Cytometry 1986;7:508-17.

van Leeuwen EF, van der Ven JTM, Engelfriet CP, von dem Borne AEGKr. Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 1982;59:23-6.

Lehrnbecher T, Foster CB, Zhu S, Leiman SF, Goldin LR, Huppi K, Chanock SJ. Variant genotypes of the low-affinity Fcγ receptors in two control populations and a review of low-affinity Fcγ receptor polymorphisms in control and disease populations. Blood 1999;94:4220-32.

Leissinger CA, Stuckey WJ. Unbound immunoglobulins are a major source of error in the quantitation of platelet surface-bound immunoglobulin levels. Transfusion 1992;32:157-61.

Levin HA, McMillan R, Tavassoli M, Longmire RL, Yelenosky R, Sacks PV. Thrombocytopenia associated with gold therapy. Observations on the mechanism of platelet destruction. Am J Med 1975;59:274-80.

Lin RY, Levin M, Nygren EN, Norman A, Lorenzana FG. Assessment of platelet antibody by flow cytometric and ELISA techninques: a comparison study. J Lab Clin Med 1990:116:479-86.

LoBuglio AF, Court WS, Vinocur L, Maglott G, Shaw GM. Immune thrombocytopenic purpura. Use of a ^{125}I-labeled antihuman IgG monoclonal antibody to quantify platelet-bound IgG. N Engl J Med 1983;309:459-63.

López JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. Blood 1998;91:4397-418.

Macchi L, Clofent-Sanchez G, Marit G, Bihour C, Durrieu-Jais C, Besse P, Nurden P, Nurden AT. PAICA: a method for characterizing platelet-associated antibodies - its application to the study of idiopathic thrombocytopenic purpura and to the detection of platelet-bound c7E3. Thromb Haemost 1996;76:1020-9.

Majerus PW. Platelets. In: Stamatoyannopoulos, Wienhuis, Majerus, Varmus, eds. The molecular basis of blood diseases, 2nd ed. Philadelphia: WB Saunders Company, 1994.

Manger K, Repp R, Spriewald BM, Rascu A, Geiger A, Wassmuth R, Westerdaal NAC, Wentz B, Manger B, Kalden JR, van de Winkel JGJ. Fcg receptor IIa polymorphism in Caucasian patients with systemic lupus erythematosus. Association with clinical symptoms. Arthritis Rheum 1998;41:1181-9.

Markovic B, Wu Z, Chesterman CN, Chong BH. Quantitation of soluble and membrane-bound FcγRIIA (CD32A) mRNA in platelets and megakaryoblastic cell line (Meg-01). Br J Haematol 1995;91:37-42.

Martin JF, Trowbridge EA. The biological significance of platelet volume: its relationship to bleeding time, platelet thromboxane B2 production and megakaryocyte nuclear DNA concentration. Thromb Res 1983;32:443-60.

Mayer JL, Beardsley DS. Varicella-associated thrombocytopenia: autoantibodies against platelet surface glycoprotein V. Pediatr Res 1996;40:615-9.

McCrae KR, Samuels P, Schreiber AD. Pregnancy-associated thrombocytopenia: pathogenesis and management. Blood 1992;80:2697-714.

McKenzie SE, Schreiber AD. Biological advances and clinical applications of Fc receptors for IgG. Curr Opin Hematol 1994;1:45-52.

McMillan R, Longmire RL, Yelenosky R, Donnell RL, Armstrong S. Quantitation of platelet-binding IgG produced in vitro by spleens from patients with idiopathic thrombocytopenic purpura. N Engl J Med 1974;812-7.

McMillan R, Pellegrino MA, Ferrone S. Correspondence. Blood 1980;55:709.

McMillan R, Tani P, Millard F, Berchtold P, Renshaw L, Woods Jr VL. Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. Blood 1987;70:1040-5.

McNeil HP, Chesterman CN, Krilis SA. Immunology and clinical importance of antiphospholipid antibodies. Adv Immunol 1991;49:193-280.

Menitove JE, Pereira J, Hoffman R, Anderson T, Fried W, Aster RH. Cyclic thrombocytopenia of apparent autoimmune etiology. Blood 1989;73:1561-9.

Mettier SR, McBride A, Li J. Thrombocytopenic purpura complicating gold therapy for rheumatoid arthritis. Blood 1948;3:1105-11.

Mezzano D, Hwang K-I, Catalano P, Aster RH. Evidence that platelet buoyant density, but not size, correlates with platelet age in man. Am J Hematol 1981;11:61-76.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215a.

Mizutani H, Furubayashi T, Kashiwagi H, Honda S, Take H, Kurata Y, Yonezawa T, Tarui S. B cells expressing CD5 antigen are markedly increased in peripheral blood and spleen lymphocytes from patients with immune thrombocytopenic purpura. Br J Haematol 1991;78:474-79.

Modderman PW, Admiraal LG, Sonnenberg A, von dem Borne AEGKr. Glycoproteins V and Ib-IX form a noncovalent complex in the platelet membrane. J Biol Chem 1992;267:364-9.

Moore A, Weksler BB, Nachman RL. Platelet Fc_{IgG} receptor: increased expression in female platelets. Thromb Res 1981;21:469-74.

Morel-Kopp M-C, Daviet L, McGregor J, Kaplan C. Drawbacks of the MAIPA technique in characterising human antiplatelet antibodies. Blood Coagul Fibrin 1996;7:144-6.

Movahed Shariat Panahi MR, Le Blean S, Schober O, Coldewey R, Deicher H. Study of platelet-associated

immunoglobulins of IgG, IgM, IgA, and IgE classes and platelet kinetics in 33 patients with idiopathic thrombocytopenic purpura. Ann Hematol 1994;69:121-8.

Mueller-Eckhardt C, Kayser W, Mersch-Baumert K, Mueller-Eckhardt G, Breidenbach M, Kugel HG, Graubner M. The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. Br J Haematol 1980;46:123-31.

Mueller-Eckhardt C, Pawelec G, Haas G, Otto C, Kiefel V, Odum N, Mueller-Eckhardt C. HLA-DP antigens in patients with chronic autoimmune thrombocytopenia (AITP). Tissue Antigens 1989;34:121-6.

Muniz-Diaz E, Madoz P, Pujol-Moix N, Pastoret C, Arilla M, Ibanez M, Guanyabens C, Domingo-Albos. Characterization of antibodies directed against platelet cryptantigens detected during the immunological study of 356 consecutive patients with presumed autoimmune thrombocytopenia (AITP). Transfus Med 1995;5:185-91.

Nagasawa T, Hasegawa Y, Komeno T, Itoh T, Ninomiya H, Abe T. Simultaneous measurements of megakaryocyte-associated IgG (MAIgG) and platelet-associated IgG (PAIgG) in chronic idiopathic thrombocytopenic purpura. Eur J Haematol 1995;54:314-20.

Najean Y, Rain JD, Billotey C. The site of destruction of autologous ^{111}In-labelled platelets and the efficiency of splenectomy in children and adults with idiopathic thrombocytopenic purpura: a study of 578 patients with 268 splenectomies. Br J Haematol 1997;97:547-50.

Nieminen U. Clinical value of a direct platelet suspension immunofluorescence test in adult idiopathic thrombocytopenic purpura. Eur J Haematol 1990;44:145-9.

Nieminen U. Immune thrombocytopenic purpura. Serological and clinical studies in patients with idiopathic, drug-induced and post-vaccination thrombocytopenia. Academic dissertation, University of Helsinki, 1992.

Nieminen U, Kekomäki R. Quinidine-induced thrombocytopenic purpura: clinical presentation in relation to drug-dependent and drug-independent platelet antibodies. Br J Haematol 1992;80:77-82.

Nomura S, Yanabu M, Kido H, Fukuroi T, Yamaguchi K, Soga T, Nagata H, Kokawa T, Yasunaga K. Antiplatelet autoantibody-related microparticles in patients with idiopathic (autoimmune) thrombocytopenic purpura. Ann Hematol 1991;62:103-7.

Nomura S, Matsuzaki T, Ozaki Y, Yamaoka M, Yoshimura C, Katsura K, Lan Xie G, Ishida T, Fukuhara S. Clinical significance of HLA-DRB1*0410 in Japanese patients with idiopathic thrombocytopenic purpura. Blood 1998;91:3616-22.

Norris CF, Surrey S, Bunin GR, Schwartz E, Buchanan GR, McKenzie SE. Relationship between Fc receptor IIA polymorphism and infection in children with sickle cell disease. J Pediatr 1996;128:813-9.

Norris CF, Pricop L, Millard SS, Taylor SM, Surrey S, Schwartz E, Salmon JE, McKenzie SE. A naturally occuring mutation in FcγRIIA: a Q to K127 change confers unique IgG binding properties to the R131 allelic form of the receptor. Blood 1998;91:656-62.

Okita JR, Pidard D, Newman PJ, Montgomery RR, Kunicki TJ. On the association of glycoprotein Ib and actin-binding protein in human platelets. J Cell Biol 1985;100:317.

Osborne JM, Chacko GW, Brandt JT, Anderson CL. Ethnic variation in frequency of an allelic polymorphism of human FcγRIIA determined with allele specific oligonucleotide probes. J Immunol Methods 1994;173:207-17.

Ouwehand W, Navarrete C. The molecular basis of blood cell alloantigens. In Molecular Haematology, ed. Provan

D, Gribben J, Blackwell Science, Oxford 2000, pp. 182-97.

Panzer S, Niessner H, Lechner K, Dudczak, Jäger U, Mayr WR. Platelet-associated immunoglobulins IgG, IgM, IgA and C3c in chronic idiopathic thrombocytopenic purpura: relation to the sequestration pattern of ^{111}Indium labelled platelets. Scand J Haematol 1986;37:97-102.

Panzer S, Penner E, Nelson PJ, Prochazka E, Benda H, Saurugger PN. Identification of the platelet glycoprotein IIb/IIIa complex as a target antigen in primary biliary cirrhosis-associated autoimmune thrombocytopenia. J Autoimmun 1990;3:473-83.

Parren PWHI, Warmerdam PAM, Boeije LCM, Arts J, Westerdaal NAC, Vlug A, Capel PJA, Aarden LA, van de Winkel JGJ. On the interaction of IgG subclasses with the low affinity FcγRIIa (CD32) on human monocytes, neutrophils, and platelets: Analysis of a functional polymorphism to human IgG2. J Clin Invest 1992;90:1537-46.

Pedersen-Bjergaard U, Andersen M, Hansen PB. Drug-specific characteristics of thrombocytopenia caused by non-cytotoxic drugs. Eur J Clin Pharmacol 1998;54:701-6.

Pereira J, Accatino L, Alfaro J, Brahm J, Hidalgo P, Mezzano D. Platelet autoantibodies in patients with chronic liver disease. Am J Hematol 1995;50:173-8.

Peterec SM, Brennan SA, Rinder HM, Wnek JL, Beardsley DS. Reticulated platelet values in normal and thrombocytopenic neonates. J Pediatr 1996;129:269-74.

Pidard D, Montgomery RR, Bennett JS, Kunicki TJ. Interaction of AP-2, a monoclonal antibody specific for the human platelet glycoprotein IIb-IIIa complex, with intact platelets. J Biol Chem 1983;258:12582-6.

Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MAM, v.d. Schoot CE, de Haas M, v.d. Borne AEGKr. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost 1998;79:1101-5.

Porges A, Bussel J, Kimberley R, Schulman I, Pollack M, Pandey J, Barandun S, Hilgartner M. Elevation of platelet associated antibody levels in patients with chronic idiopathic thrombocytopenic purpura expressing the B8 and/or DR3 allotypes. Tissue Antigens 1985;26:132-7.

Prandini MH, Denarier E, Frachet P, Uzan G, Marguerie G. Isolation of the human platelet glycoprotein IIb gene and characterization of the 5' flanking region. Biochem Biophys Res Commun 1988;156:595-601.

Pujol M, Rebera A, Vilardell M, Ordi J, Feliu E. High prevalence of platelet autoantibodies in patients with systemic lupus erythematosus. Br J Haematol 1995;89:137-41.

Reid DM, Jones CE, Vostal JG, Shulman NR. Western Blot identification of platelet proteins that bind normal serum immunoglobulins. Characteristics of a 95-Kd reactive protein. Blood 1990;75:2194-203.

Reid DM, Jones CE, Luo C, Shulman NR. Immunoglobulins from normal sera bind platelet vinculin and talin and their proteolytic fragments. Blood 1993;81:745-51.

Reilly AF, Norris CF, Surrey S, Bruchak FJ, Rappaport EF, Schwarzt E, McKenzie SE. Genetic diversity in human Fc receptor II for immunoglobulin G: Fcγ receptor IIA ligand-binding polymorphism. Clin Diagn Lab Immun 1994;1:640-4.

Richards EM, Baglin TP. Quantitation of reticulated platelets: methodology and clinical application. Br J Haematol 1995;91:445-51.

Rinder HM, Munz UJ, Ault KA, Bonan JL, Smith BR. Reticulated platelets in the evaluation of thrombopoietic disorders. Arch Pathol Lab Med 1993;117:606-10.

Rinder HM, Bonan JL, Anandan S, Rinder CS, Rodriques PA, Smith BR. Noninvasive measurement of platelet kinetics in normal and hypertensive pregnancies. Am J Obstet Gynecol 1994;170:117-22.

Rizvi MA, Shah SR, Raskob GE, George JN. Drug-induced thrombocytopenia. Curr Opin Hematol 1999;6:349-53.

Robinson MSC, Mackie IJ, Khair K, Liesner R, Goodall AH, Savidge GF, Machin SJ, Harrison PH. Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-labelling of dense granules by fluorescent dyes. Br J Haematol 1998;100:351-7.

Romp KG, Peters WP, Hoffman M. Reticulated platelet counts in patients undergoing autologous bone marrow transplantation: an aid in assessing marrow recovery. Am J Hematol 1994;46:319-24.

Rosenfeld SI, Looney RJ, Leddy JP, Phipps DC, Abraham GN, Anderson CL. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes. J Clin Invest 1985;76:2317-22.

Rosenfeld CS, Nichols G, Bodensteiner DC. Flow cytometric measurement of antiplatelet antibodies. Am J Clin Pathol 1987a;87:518-22.

Rosenfeld SI, Ryan DH, Looney RJ, Anderson CL, Abraham GN, Leddy JP. Human Fcγ receptors: stable inter-donor variation in quantitative expression on platelets correlates with functional responses. J Immunol 1987b;138:2869-73.

Rubinstein E, Kouns WC, Jennings LK, Boucheix C, Carroll RC. Interaction of two GPIIb/IIIa monoclonal antibodies with platelet Fc receptor (FcγRII). Br J Haematol 1991;77:80-6.

Sainio S, Joutsi L, Järvenpää A-L, Kekomäki R, Koistinen E, Riikonen S, Teramo K. Idiopathic thrombocytopenic purpura in pregnancy. Acta Obstet Gynecol Scand 1998;77:272-7.

Salmon JE, Edberg JC, Brogle NL, Kimberley RP. Allelic polymorphisms of human Fcγ receptor IIA and Fcγ receptor IIIB. Independent mechanisms for differences in human phagocyte function. J Clin Invest 1992;89:1274-81.

Salmon JE, Millard S, Schachter LA, Arnett FC, Ginzler EM, Gourley MF, Ramsey-Goldman R, Peterson MGE, Kimberley RP. FcγRIIA alleles are heritable risk factors for lupus nephritis in African Americans. J Clin Invest 1996;97:1348-54.

Sanders LAM, van de Winkel JGJ, Rijkers GT, Voorhorst-Ogink MM, de Haas M, Capel PJA, Zegers BJM. Fcγ receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J Infect Dis 1994;170:854-61.

Saxon BR, Blanchette VS, Butchart S, Lim-Yin J, Poon AO. Reticulated platelet counts in the diagnosis of acute immune thrombocytopenic purpura. J Pediatr Hematol Oncol 1998;20:44-8.

Shulman NR, Marder VJ, Weinrach RS. Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Physiologic, serologic and isotopic studies. Ann NY Acad Sci 1965;124:499-542.

Silvestri F, Virgolini L, Savignano C, Zaja F, Velisig M, Baccarani M. Incidence and diagnosis of EDTA-

dependent pseudothrombocytopenia in a consecutive outpatient population referred for isolated thrombocytopenia. Vox Sang 1995;68:35-9.

Singal DP, Reid B, Green D, D´Souza, Bensen WG, Buchanan WW. Polymorphism of major histocompatibility complex extended haplotypes bearing HLA-DR3 in patients with rheumatoid arthritis with gold-induced thrombocytopenia or proteinuria. Ann Rheum Dis 1990;49:582-6.

Sinha AA, Lopez MT, McDevitt HO. Autoimmune diseases: the failure of self tolerance. Science 1990;248:1380-8.

Smyth LJC, Snowden N, Carthy D, Papasteriades C, Hajeer A, Ollier WER. FcγRIIa polymorphism in systemic lupus erythematosus. Ann Rheum Dis 1997;56:744-6.

Song KS, Kim BS, Choi CR, Lee SM. Association of Br polymorphism of platelet GP Ia gene and immune thrombocytopenic purpura. Platelets 1997;8:361-4.

Soulier JP, Patereau C, Drouet J. Platelet indirect radioactive Coombs test. Its utilization for Pla1 grouping. Vox Sang 1975;29:253-68.

Stasi R, Stipa E, Masi M, Cecconi M, Scimo MT, Oliva F, Sciarra A, Perrotti A, Adorno G, Amadori S, Papa G. Long-term observation of 208 adults with chronic idiopathic thrombocytopenic purpura. Am J Med 1995;98:436-42.

Stavem P, Stromme J, Bull O. Immunological studies in a case of gold salt induced thrombocytopenia. Scand J Haematol 1968;5:271-7.

Stockelberg D, Hou M, Jacobsson S, Kutti J, Wadenvik H. Evidence for a light chain restriction of glycoprotein Ib/IX and IIb/IIIa reactive antibodies in chronic idiopathic thrombocytopenic purpura (ITP). Br J Haematol 1995;90:175-9.

Stohlawetz P, Schulenburg A, Stiegler G, Panzer S, Höcker P, Kahls P, Schwarzinger I, Müllauer L, Simonitsch I, Geissler K, Jilma B. The proportion of reticulated platelets is higher in bone marrow than in peripheral blood in haematological patients. Eur J Haematol 1999;63:239-44.

Stricker RB, Shuman MA. Quinidine purpura: evidence that glycoprotein V is a target platelet antigen. Blood 1986;67:1377-81.

Szatkowski NS, Kunicki TJ, Aster RH. Identification of glycoprotein Ib as a target autoantibody in idiopathic (autoimmune) thrombocytopenic purpura. Blood 1986;67:310-5.

Taaning E, Petersen S. Pattern of platelet-associated immunoglobulin (classes and IgG subclasses) in childhood immune thrombocytopenic purpura. Eur J Haematol 1988;41:449-53.

Takada Y, Hemler ME. The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. J Cell Biol 1989;109:397-407.

Tani P, Berchtold P, McMillan R. Autoantibodies in chronic ITP. Blut 1989;59:44-6.

Tarallo P, Humbert J-C, Mahassen P, Fournier B, Henny J. Reticulocytes: biological variations and reference limits. Eur J Haematol 1994;53:11-5.

Tarantino D, Kunicki TJ, Nugent DJ. The estrogen receptor is present in human megakaryocytes. Ann NY Acad Sci 1994;714:293-6.

Tàssies D, Reverter JC, Cases A, Escolar G, Villamor N, López-Pedret J, Castillo R, Ordinas A. Reticulated platelets in uremic patients: effect of hemodialysis and continuous ambulatory peritoneal dialysis. Am J Hematol 1995;50:161-6.

Thorsby E. HLA associated diseases. Hum Immunol 1997;53:1-11.

Thude H, Gatzka E, Anders O, Barz D. Allele frequencies of human platelet antigen 1, 2, 3, and 5 systems in patients with chronic refractory autoimmune thrombocytopenia and in normal persons. Vox Sang 1999;77:149-53.

Tijhuis GJ, Klaassen RJL, Modderman PW, Ouwehand WH, von dem Borne AEGKr. Quantification of platelet-bound immunoglobulins of different class and subclass using radiolabelled monoclonal antibodies: assay conditions and clinical application. Br J Haematol 1991;77:93-101.

Tomer A, Schreiber AD, McMillan R, Cines DB, Burstein SA, Thiessen AR, Harker LA. Menstrual cyclic thrombocytopenia. Br J Haematol 1989;71:519-24.

Tomiyama Y, Kurata Y, Mizutani H, Kanakura Y, Tsubakio T, Yonezawa T, Tarui S. Platelet GP IIb as a target antigen in two patients with chronic idiopathic thrombocytopenic purpura. Br J Haematol 1987;66:535-8.

Tomiyama Y, Kurata Y, Shibata Y, Honda S, Furubayashi T, Mizutani H, Tsubakio T, Yonezawa T, Tarui S. Immunochemical characterization of an autoantigen on platelet glycoprotein IIb in chronic ITP: comparison with the Bak^a alloantigen. Br J Haematol 1989;71:77-83.

Tomiyama Y, Take H, Honda S, Furubayashi T, Mizutani H, Tsubakio T, Kurata Y, Yonezawa T, Tarui S. Demonstration of platelet antigens that bind platelet-associated autoantibodies in chronic ITP by direct immunoprecipitation procedure. Br J Haematol 1990;75:92-8.

Tomiyama Y, Kekomäki R, McFarland, Kunicki TJ. Antivinculin antibodies in sera of patients with immune thrombocytopenia and in sera of normal subjects. Blood 1992;79:161-8.

Tsubakio T, Tani P, Woods VL Jr, McMillan R. Autoantibodies against platelet GPIIb/IIIa in chronic ITP react with different epitopes. Br J Haematol 1987;67:345-8.

Visentin GP, Newman PJ, Aster RH. Characteristics of quinine- and quinidine-induced antibodies specific for platelet glycoproteins IIb and IIIa. Blood 1991;77:2668-76.

van Vliet HHDM, Kappers-Klunne MC, van der Hel JWB, Abels J. Antibodies against glycosphingolipids in sera of patients with idiopathic thrombocytopenic purpura. Br J Haematol 1987;67:103-8.

Warkentin TE, Hayward CPM, Smith CA, Kelly PM, Kelton JG. Determinants of donor platelet variability when testing for heparin-induced thrombocytopenia. J Lab Clin Med 1992;120:371-9.

Warkentin TE, Elavathil LJ, Hayward CPM, Johnston MA, Russett JI, Kelton JG. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. Ann Intern Med 1997;127:804-12.

Warmerdam PAM, van de Winkel JGJ, Gosselin EJ, Capel PJA. Molecular basis for a polymorphism of human Fcγ receptor II (CD32). J Exp Med 1990;172:19-25.

Warmerdam PAM, van de Winkel JGJ, Vlug A, Westerdaal NAC, Capel PJA. A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. J Immunol 1991;147:1338-43.

Warner MN, Moore JC, Warkentin TE, Santos AV, Kelton JG. A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura. Br J Haematol 1999; 104:442-7.

Watanabe K, Takeuchi K, Kawai Y, Ikeda Y, Kubota F, Nakamoto H. Automated measurement of reticulated platelets in estimating thrombopoiesis. Eur J Haematol 1995;54:163-71.

Wenger RH, Kieffer N, Wicki AN, Clemetson KJ. Structure of the human blood platelet membrane glycoprotein Ibα gene. Biochem Biophys Res Commun 1988;156:389-95.

Williams Y, Lynch S, McCann S, Smith O, Feighery C, Whelan A. Correlation of platelet FcγRIIA polymorphism in refractory idiopathic (immune) thrombocytopenic purpura. Br J Haematol 1998;101:779-82.

Winiarski J. Antibodies to platelet membrane glycoprotein antigens in three cases of infectious mononucleosis-induced thrombocytopenic purpura. Eur J Haematol 1989;43:29-34.

Woods VL Jr, Oh EH, Mason D, McMillan R. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. Blood 1984a;63:368-75.

Woods VL Jr, Kurata Y, Montgomery RR, Tani P, Mason D, Oh EH, McMillan R. Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood 1984b;64:156-60.

Wu Z, Markovic B, Chesterman CN, Chong BH. Characterization of Fcγ receptors on human megakaryocytes. Thromb Haemost 1996;75:661-7.

Yagi M, Edelhoff S, Disteche CM, Roth GJ. Human platelet glycoprotiens V and IX: mapping of two leucine-rich glycoprotein genes to chromosome 3 and analysis of structures. Biochem 1995;34:16132-7.