SOME PROPERTIES OF PLATELET ANTIBODIES

WITH SPECIAL REFERENCE TO THE AUTOANTIBODIES IN CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

By
Dick Stockelberg

Göteborg 1996

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I. Stockelberg D, Hou M, Jacobsson S, Kutti J, Wadenvik H. Detection of platelet antibodies in chronic idiopathic thrombocytopenic purpura (ITP). A comparative study using flow cytometry, a whole platelet ELISA and an antigen capture ELISA. **Eur J Haematol 1996: 56: 72-77**

II. 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: 175-179**

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IV. Hou M, Stockelberg D, Kutti J, Wadenvik H. Glycoprotein IIb/IIIa autoantigenic repertoire in chronic idiopathic thrombocytopenic purpura. **Br J Haematol 1995: 91: 971-975**

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Thesis defended May 8, 1996

Abstract: Since the pivotal discovery by Harrington and coworkers in 1951 it is well recognized that a serum factor, subsequently localized to the immunoglobulin fraction, is involved in the pathogenesis of chronic idiopathic thrombocytopenic purpura (ITP). Over the years, much effort was devoted to the development of techniques for the detection of this platelet antibody. Assays measuring the total platelet immunoglobulin content were shown to be neither specific nor sensitive enough. In the early 1980:s it became evident that certain platelet membrane glycoproteins (GPs) were targeted by autoantibodies, alloantibodies and carried blood group ABO antigens. As a consequence thereof refined and antigen-specific assays were developed. Currently, few data are available regarding the epitopes on the GPs acting as autoantigens. Nevertheless, it has been hypothesized that the autoantibodies in chronic ITP are clonally derived. The purpose of the present study was to: (a) evaluate different techniques, suitable for the routine laboratory, for detection of platelet antibodies in patients with ITP, (b) explore the hypothesis of clonally derived autoantibodies in ITP, and (c) characterize blood group A antigen expressing platelet GPs. Blood and serum samples were obtained from patients with an unequivocal diagnosis of chronic ITP. Employing whole platelet based assays (whole platelet ELISA and flow cytometry) platelet antibodies were detected in approximately 35% of the ITP patients, and the platelet antibodies were most frequently found to belong to the IgG immunoglobulin class. Using an antigen specific assay, the modified antigen capture ELISA (MACE), antibodies specific for GPIIb/IIIa and/or GPIb/IX were recorded in about one third of the ITP patients. However, there was a discrepancy as to results between the assays. Due to low sensitivity none of the assays can be recommended for routine clinical use in the differential diagnosis of thrombocytopenias. The results obtained support the hypothesis of clonally derived autoantibodies in chronic ITP. Thus, first, we found that these autoantibodies, in the majority of patients, were light chain restricted; second, IgG-F(ab´)$_2$ fragments prepared from two prototype ITP patients with known anti-GPIIb/IIIa were each able to block the binding of serum IgG to GPIIb/IIIa in at least 50% of other ITP patients, favoring a homogeneous autoepitope repertoire. However, an expanded clonal B-cell population could not be detected by using flow cytometric "clonal excess" analysis or PCR-analysis of Ig-gene rearrangements (CDR3). Apart from the previously reported blood group A-antigen expressing platelet GPs, i.e. GPIb, GPIIa, GPIIb, GPIIIa and PECAM, we observed that an anti-A antibody immunoblotted and immunoprecipitated some other uncharacterized GPs which had a molecular weight of 70-90 kD. These latter platelet proteins were characterized by us as platelet GPIV and GPV.

Key words: Idiopathic thrombocytopenic purpura; Autoantibody; Glycoprotein; Epitope; F(ab´)$_2$; ELISA; Flow cytometry; B-cells; Clonality; Blood group antigens

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"Morbus maculosus haemorrhagicus"

An adult girl, without manifest cause, was attacked recently, towards the period of her menses, with a sudden severe hemorrhage from the nose, with bright but foul blood escaping together with a bloody vomiting of a very thick extremely black blood. Immediately there appeared about the neck and on the arms, spots partly black, partly violaceous or purple, such as are often seen in malignant smallpox...; moreover the number of the spots increasing and surrounding completely both of the eyes, the back of the nose and the skin around the mouth and chin, with a livid black color, like marked from bruises

Opera Medica, Paul Gottlieb Werlhof, 1776

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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CONTENTS

INTRODUCTION

Clinical features

In his book *Opera Medica*, published in 1776, Paul Gottlieb Werlhof devoted a chapter to "Morbus maculosus haemorrhagicus", which he first described forty years earlier (Werlhof, 1776). A profound diminution in platelet count in Werlhof´s disease was first recognized by Krauss (1883), and since the 1950s, it has been recognized that platelets can be destroyed by immunologic processes. Antiplatelet antibodies can target at alloantigens, autoantigens, and drug-induced neoantigens (Aster, 1989); high titer ABO antibodies can also cause refractoriness to ABO incompatible platelet transfusions (Aster, 1965; Duquesnoy *et al,* 1979; Skogen *et al,* 1988). Immune mechanisms involving the platelet-specific alloantigens are seen in two distinct clinical entities, neonatal alloimmune thrombocytopenic purpura (NATP) and posttransfusion purpura (PTP). In NATP destruction of the fetal platelets is caused by transplacentally acquired maternal antibodies directed against platelet-specific alloantigens inherited from the father and lacking in the mother (Aster, 1989); an incompatibility in the diallelic alloantigen HPA-1 ($Pl^{A1/A2}$) or HPA-5 ($Br^{a/b}$) is most commonly seen (Mueller-Eckhardt *et al,* 1989). PTP, also caused by platelet-specific alloantibodies, occurs about a week after a red cell or platelet transfusion (Aster, 1989; Taaning & Svejgaard, 1994). This disease is typically seen in an elderly multiparous and HPA-1a-negative (Pl^{A1}-negative) woman, who previously has been transfused with blood products. However, these two conditions are rare and idiopathic thrombocytopenic purpura (ITP), caused by autoantibodies directed against certain platelet antigens, is the most common entity of the immune thrombocytopenias. ITP is an acquired disorder and can affect both children and adults. However, the clinical syndromes of ITP are distinct between children and adults. Childhood ITP characteristically is acute in onset, occurs within one or two weeks of an infection usually of viral origin, and resolves spontaneously within six months - acute ITP. Adult ITP typically has an insidious onset and rarely resolves spontaneously - chronic ITP (George et al, 1995).

Chronic ITP is a diagnosis made by exclusion, and the following diagnostic criteria are frequently applied: (1) thrombocytopenia (platelet count $<100 \times 10^9/l$) for at least six

months, (2) no known underlying disease, e.g. lymphomas, systemic lupus erythematosus, or HIV-infection, (3) normal or increased number of megakaryocytes in a bone marrow biopsy, and (4) a normal spleen size (Karpatkin, 1980). The incidence of chronic ITP among adults is approximately 6/100,000, women are affected more frequently than men (3:1), and 70 percent of the women are less than 40 years old (George *et al,* 1994; George *et al,* 1995). The clinical picture can sometimes be dramatic with severe bleedings from skin and mucous membranes, and the platelet count is not corrected by platelet transfusions. Frequently, corticosteroid treatment will raise the platelet count to normal values within 1-2 weeks; deaths from severe hemorrhages are rare but may occur especially in elderly patients (Pizzuto & Ambriz, 1984; Cortelazzo *et al,* 1991). Complete and sustained remissions (CR) are, however, only obtained in 30% of patients treated with corticosteroids (for review see: George *et al,* 1995). Other treatment modalities, i.e. high dose intravenous immunoglobulins (IVIg) and cytostatic drugs, have been shown to be effective in chronic ITP (for review see: George *et al,* 1995). Following splenectomy, complete and sustained remissions will be obtained in approximately 65% of the patients with chronic ITP; over one third of the splenectomized patients do not obtain a complete remission, and many of them will require continuous medical treatment (George *et al,* 1994; George *et al,* 1995).

Pathophysiology

In the 1950s, the term idiopathic thrombocytopenic purpura referred to a clinical disorder of unknown etiology associated with thrombocytopenia and purpura. It was reported that mothers with ITP often gave birth to children who developed transient thrombocytopenia, suggesting the transfer of a humoral factor (Robson & Davidsson, 1950; Epstein *et al,* 1950). Harrington and coworkers reported in 1951 direct evidence for the existence of a serum factor responsible for the thrombocytopenia in ITP; when plasma from patients with ITP was given intravenously to healthy volunteers or patients with inoperable malignant neoplasms, a marked and transient thrombocytopenia was seen in more than half of the recipients (Harrington *et al,* 1951). The factor responsible for the platelet destruction was subsequently shown to carry many characteristics of an antibody, i.e. it was present in the Ig-fraction of serum, it was absorbed from serum by normal platelets, and the degree of platelet destruction was proportional to the amount of serum infused (Shulman *et al,* 1965).

Increasing doses of ITP plasma transfused to healthy recipients aggravated the thrombocytopenia; when splenectomized recipients were transfused much higher doses were required, suggesting that splenic removal, rather than vascular destruction was of importance (Shulman *et al*, 1965). Also, when ITP plasma was given during steroid protection, a less pronounced thrombocytopenia was elicited.

The spleen is considered to be a major site of antiplatelet antibody production in chronic ITP. It has been reported that the spleen in ITP displays lymphoid hyperplasia and alterations in the microcirculation (Tavasolli & McMillan, 1975; Schmidt *et al*, 1991). In chronic ITP platelet kinetic studies using ^{51}Cr- or ^{111}In-labelled platelets have, apart from a profoundly shortened platelet mean life span, demonstrated that the spleen is the major site for platelet destruction (Aster & Keene, 1969; Harker & Finch, 1969; Branehög *et al*, 1974; Ballem *et al*, 1987; Siegel *et al*, 1989). The platelet production rate is most commonly elevated, as reflected by an increased bone marrow megakaryocyte number and size (Branehög *et al*, 1975). However, it has been reported that the platelet production rate may be reduced in some cases of ITP, possibly due to the effect of platelet autoantibodies on the megakaryocytes (McMillan *et al*, 1978; Hoffman *et al*, 1985; Stoll *et al*, 1985; Heyns *et al*, 1986; Ballem *et al*, 1987)

Antiplatelet antibodies

Early assays for platelet antibody detection were designed to measure the effect of patient plasma on the function of normal platelets, e.g. induction of platelet aggregation, secretion and lysis. However, these tests were insensitive to the abnormalities specific for ITP (Jackson *et al*, 1963; Aster & Enright, 1969; Hirschman & Shulman, 1973). Studies aimed at quantification of platelet associated IgG (PAIgG) opened a new era in the investigation of immune thrombocytopenias (McMillan *et al*, 1971). High PAIgG values were found in patients with ITP, and it was assumed that all platelet IgG was located on the platelet surface and constituted antiplatelet antibody (Dixon *et al*, 1975; Luiken *et al*, 1977). Further studies, however, showed that normal platelets contain two distinct pools of IgG, one surface located and one intracellular. The surface oriented pool has been estimated to contain about 100 molecules of IgG. Intracellularly, however, approximately 20,000 IgG molecules are

stored in the α-granules (George, 1989; George, 1990). The original techniques used for the detection of PAIgG measured the total platelet IgG, i.e. the α-granule IgG-content was included, and the data obtained were inaccurately assumed to be a measure of platelet surface IgG (Kelton *et al,* 1979; Kelton *et al,* 1980; Tsubakio *et al,* 1981).

PAIgG

Total platelet IgG is increased in ITP, and is inversely related to the platelet count, i.e. the highest values are seen in the most severe thrombocytopenias (Kelton *et al,* 1982; George, 1990). On the other hand, also patients with non-immune thrombocytopenias demonstrated elevated levels of total platelet IgG (Kelton *et al,* 1982; Kelton *et al,* 1989). Indeed, it has been shown that nearly all thrombocytopenic platelets contain more IgG, IgA, IgM and albumin than normal platelets, and that the increased PAIgG might be related to an increased platelet volume and enhanced thrombopoietic stimulation (Dixon *et al,* 1975; Luiken *et al,* 1977; Zeigler *et al,* 1978; Hegde *et al,* 1981; Nel *et al,* 1983; George & Saucerman, 1988; Hattori *et al,* 1992). Such platelets are larger and contain more IgG when the thrombocytopenia is severe; these variables return to normal values when the platelet count is normalized. This is true for all patients with an increased platelet production, but is not seen in patients with thrombocytopenia caused by bone marrow failure (George, 1990). An increased PAIgG is also observed in disorders with elevated plasma IgG concentrations, e.g. multiple myeloma, liver diseases, and chronic inflammatory/infectious diseases; in these conditions the increased PAIgG mainly reflects an increased plasma IgG (McGrath *et al,* 1979; Landolfi *et al,* 1980; Barrison *et al,* 1981; George & Saucerman, 1988), and not an enhanced platelet turnover.

Platelet surface IgG

Platelet surface IgG was measured directly by the binding of a ^{125}I-labelled monoclonal antibody to the Fc-region of all subclasses of IgG (LoBuglio *et al,* 1983; Court *et al,* 1987; George, 1991) or by ^{125}I-labelled staphylococcal protein A (Shaw *et al,* 1984). By using these techniques it has been clearly shown that even normal platelets have surface oriented IgG, but the origin of this IgG is unknown (Court *et al,* 1987). In ITP, platelet surface IgG is always increased and the level is higher in acute non-treated

patients than in patients with chronic refractory ITP; the levels can also fluctuate in the individual patient during the course of the disease (Cines & Schreiber 1979; Court *et al,* 1987; George, 1990). In many patients with thrombocytopenia of non-immune etiology or due to bone marrow failure, platelet surface IgG is also increased (Shaw *et al,* 1984; Court *et al,* 1987; George, 1988; Kelton *et al,* 1989; George, 1990; George, 1991).

Antibodies to surface glycoproteins.

In 1982 van Leeuwen and collaborators provided the first evidence for autoantibody binding to certain platelet glycoproteins in chronic ITP (van Leeuwen *et al,* 1982). They used the platelet suspension immunofluorescence test (PSIFT) and found that platelet eluted antibodies bound to normal but not to thrombasthenic platelets in 32 out of 42 patients with chronic ITP. Thrombasthenic platelets are known to lack the glycoprotein (GP) IIb/IIIa complex, and the authors suggested that ITP patients had autoantibodies targeting one of these glycoproteins (van Leeuwen *et al,* 1982). Woods et al (1984a; 1984b), using a microtiter well assay, demonstrated the binding of autoantibodies from ITP patients to the GPIIb/IIIa or GPIb/IX complexes. Their observations were further confirmed by immunoprecipitation. In addition, Beardsley et al (1984), using an immunoblot assay, demonstrated autoantibody binding to GPIIIa in plasma of children with chronic ITP. Two novel assays were reported in 1987, the immunobead assay (McMillan *et al,* 1987) and the monoclonal antibody specific immobilization of platelet antigens (MAIPA) assay (Kiefel *et al,* 1987). These two assays have the advantage of allowing the measurement of both platelet-associated and plasma autoantibodies against platelet GPs, while the prior assays were limited to the detection of only plasma autoantibodies. Using these techniques, GPIIb/IIIa and GPIb/IX have been the most frequently encountered autoantigens in chronic ITP (McMillan *et al,* 1987; Kiefel *et al,* 1991; Fujisawa *et al,* 1993a). However, autoantibodies binding to GPIa/IIa and GPIV have been described (He *et al,* 1994). A direct assay of platelet-associated glycoprotein-specific autoantibodies are considered more frequently to yield a positive result in ITP compared to assays of plasma antibodies (McMillan *et al,* 1987; Kokawa *et al,* 1993). Moreover, the titers of the glycoprotein-specific autoantibodies have been reported to vary inversely with the degree of thrombocytopenia during the course of the disease (Berchtold & Wenger,

1993). Also, the antibody titer was shown to decrease in association with improved platelet counts following treatment with corticosteroids, splenectomy, and cyclophosphamide; no change in antibody titer was seen with vincristine and danazol therapy, despite an increase in platelet count (Fujisawa *et al,* 1993b).

Glycoprotein autoantigenic repertoire

A few previous studies have addressed the repertoire of autoantigenic epitopes on GPIIb/IIIa by analyzing the competitive binding between human autoantibodies and murine monoclonal antibodies (MoAbs) (Varon & Karpatkin, 1983; Tsubakio *et al,* 1987; Fujisawa *et al,* 1990). Also, enzyme cleaved GPIIb or IIIa fragments, and synthetic peptides corresponding to different sequences of GPIIIa have been used to localize epitopes on the respective glycoprotein (Tomiyama *et al,* 1989; Fujisawa *et al,* 1991; Kekomaki *et al,* 1991; De Souza *et al,* 1992; Fujisawa *et al,* 1992). Moreover, GP specific human MoAbs have been developed as important tools in the search for GP autoepitopes in chronic ITP; until now only a few cryptic epitopes on GPIIb/IIIa have been recognized using such antibodies (Nugent, 1987; Kunicki *et al,* 1990; Kunicki *et al,* 1991). Despite the fact that a few investigators (Tomiyama *et al,* 1989; Fujisawa *et al,* 1991; Kekomaki *et al,* 1991; De Souza *et al,* 1992; Fujisawa *et al,* 1992) have been successful in localizing certain autoantigenic epitopes to regions of GPIIb or GPIIIa, the blocking experiments using murine MoAbs have provided contradictory data regarding the homogeneity of the GPIIb/IIIa autoantigenic repertoire (Varon & Karpatkin, 1983; Tsubakio *et al,* 1987; Fujisawa *et al,* 1990). Kekomaki et al (1991) found that a 33 kD chymotryptic core fragment of GPIIIa was a frequent target for autoantibodies in chronic ITP. Moreover, in a study by Nugent and coworkers (1989) it was shown that the repertoire of idiotypes expressed by human antibodies specific for platelet membrane glycoproteins in chronic ITP could be narrowly defined, mimicking a clonal restriction. Further support for the hypothesis of clonal autoantibodies in ITP were provided by Christie et al (1993) and van der Harst et al (1990). These investigators reported that antibodies in immune thrombocytopenias were light chain restricted and that expanded clonal B-cell populations were commonly present.

Cellular immunity

Abnormal production of autoantibodies in autoimmune states may be due to intrinsic defects within the B-lymphocyte populations or due to aberrant mechanisms which indirectly influence the B-cells. Abnormalities in cellular immunity have previously been reported in a number of autoimmune diseases, and multiple lymphocyte defects are known to be present in chronic ITP (Hymes & Karpatkin, 1990; Semple & Freedman, 1992). Most studies have been performed on T-cell abnormalities and contradicting results have often been obtained; several investigators have reported a decrease in CD8+ T-suppressor lymphocytes and changes in the CD4+/CD8+ ratios (Trent *et al,* 1981; Lauria *et al,* 1981; Peller *et al,* 1986;), whereas others have reported normal CD4+/CD8+ ratios and increases in CD4+ and CD8+ T-lymphocytes (Lauria *et al,* 1983; Scott *et al,* 1983; Mizutani *et al,* 1985; Semple & Freedman, 1991). Mizutani et al (1991) reported an increase in circulating and splenic CD5+ B-lymphocytes in patients with ITP, and Semple & Freedman (1991) found an increase in CD19+ B-cells, among which CD5+ B-cells are a subset. A T-suppressor cell (CD8+) hyporesponsiveness, with concomitant increase in autoreactive T-helper cells (Hymes & Karpatkin, 1990; Semple & Freedman, 1991), may be responsible for an inappropriate autoantibody production. Based on flow cytometric analysis, two independent groups of investigators have also demonstrated expanded clonal B-cell populations in immune thrombocytopenias (van der Harst *et al,* 1990; Christie *et al,* 1993). However, these clonal expansions were not restricted to CD5+ B-cells.

Blood group ABH antigens

ABH antigens are carbohydrates being attached to oligosaccharide core moieties on glycolipids and glycoproteins (Watkins, 1980). Also, it is well recognized that human platelets carry blood group ABH antigens both as extrinsic antigens passively adsorbed from plasma and as intrinsic antigens (Dunstan *et al,* 1985; Dunstan & Mansbach, 1985). Impaired platelet recovery remains to be a problem in the transfusion of ABO-incompatible platelets (Aster, 1965; Duquesnoy *et al,* 1979; Brand *et al,* 1986; Heal *et al,* 1987; Ogasawara *et al,* 1993). The ABH-expressing glycoproteins carry glycan in two different ways; glycosylation may occur at asparagine sites by linking D-β-N-acetylglucosamine to the amino group (*N*-glycan), or at serine or threonine sites by linking D-α-acetylgalactosamine through the

hydroxyl group (*O*-glycan) (Clausen & Hakomori, 1989). The protein based expression of blood group ABH determinants on platelets has been assigned to the GPs Ib, IIa, IIb, IIIa, and PECAM (Santoso *et al,* 1991; Ogasawara *et al,* 1993; Santoso *et al,* 1993; Hou *et al,* 1996). Most recently, it was reported by our group that ABH antigens were expressed on some other uncharacterized glycoproteins, having a molecular weight of 70-90 kD; with respect to the molecular weight we considered GPIV and GPV as likely candidates (Hou *et al,* 1996). Indeed, GPIV and GPV are major platelet membrane GPs, with approximately 20,000 and 11,000 copies, respectively, on a resting platelet (Legrand *et al,* 1991; Modderman *et al,* 1992). The numbers of the potential glycosylation sites of GPIV and GPV have been elucidated by the complete cDNA-deduced protein sequences and by compositional analysis of the purified glycoproteins (Oquendo *et al,* 1989; Lanza *et al,* 1993); GPIV and GPV were found to have as many as ten and eight potential *N*-glycosylation sites, respectively. Hence, it is theoretically suggestive that these two glycoproteins should contain ABH blood group determinants.

AIMS OF THE INVESTIGATION

The objectives of the present study were:

to evaluate different techniques suitable for large scale screening of serum platelet antibodies

to explore the hypothesis of oligoclonal/clonal autoantibodies in chronic ITP

to examine the homogeneity of autoantigen epitopes on GPIIb/IIIa in chronic ITP

to further characterize the 70-90 kD, blood group A antigen expressing platelet membrane glycoproteins

MATERIAL AND METHODS

I. Patients and controls

Sera from patients with chronic ITP were the core of the study. The diagnosis of chronic ITP was based on the following findings: a thrombocytopenia persisting for more than 6 months, normal or increased numbers of megakaryocytes in a bone marrow biopsy, normal spleen size, and no other known causes of the thrombocytopenia. Further clinical characteristics for the patients are given in the respective papers.

Sera from healthy blood donors with no history of blood transfusions or pregnancy served as negative controls.

An anti-HPA-1a (anti-PI^{A1}) serum was obtained from a HPA-1a-negative (PI^{A1}-negative) woman who had been treated with buffy coat transfusions because of repeated miscarriages; at two subsequent pregnancies she gave birth to babies with neonatal alloimmune thrombocytopenia.

All sera were stored at -70° C until used in the assays

II. Platelets

Platelets from healthy blood group O donors were isolated from EDTA-anticoagulated whole blood by differential centrifugation and washed twice with 0.01 M phosphate buffered saline (PBS), pH 7.4, containing 10 mM EDTA. The platelet counts were determined using an automatic cell analyzer (H2, Technicon, USA).

III. Platelet lysate

Washed platelets (1×10^8) were resuspended in 90 µl 50 mM Tris, 145 mM NaCl buffer (TBS), pH 7.5, mixed with 10 µl 10% Triton X-100, and agitated for 30 minutes at 4° C. The solubilized platelets were then centrifuged for 30 minutes at 26,000 g to pellet the insoluble material. The supernatant platelet lysate was stored frozen at -70° C until used as a source of soluble platelet proteins.

IV. Biotinylation of platelet membrane proteins (paper V)

Instead of the commonly used radiolabelling techniques, we employed a non-radioactive method for cell surface labelling. This technique has previously been described by other investigators (Fabris *et al*, 1992; Smith *et al*, 1993). Briefly, washed platelets, at a concentration of 1 x 10^9/ml, were incubated with 5 mM NHS-LC-Biotin (Pierce, USA), in 0.01 M PBS/EDTA, pH 7.4, for 30 min at ambient temperature. The platelets were then washed three times with PBS/EDTA and resuspended in the same buffer.

V. Purification of IgG and IgG-F(ab´)$_2$ fragments (paper IV)

IgG was purified from 1 ml aliquotes of patients and normal sera by affinity chromatography on a protein A-sepharose column. Following elution from the column IgG was extensively dialyzed with 0.01 M PBS, pH 7.4. IgG-F(ab´)$_2$ fragments were prepared from the purified IgG by solid phase pepsin digestion according to the manufacturer's instructions (F(ab´)$_2$ preparation kit, Pierce, USA). Undigested IgG and large Fc fragments were absorbed by protein A chromatography, and small Fc fragments removed by dialysis. F(ab´)$_2$ fragments were verified by molecular weight analysis, using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, showing a single band at 120 kD.

VI. IgG-F(ab´)$_2$ blocked and unblocked platelets (paper IV)

These platelets were used to evaluate whether IgG-F(ab´)$_2$ fragments from one ITP-patient were able to inhibit the platelet binding of autoantibodies from other patients with chronic ITP. IgG-F(ab´)$_2$ fragments were prepared from healthy volunteers, and from two prototype ITP patients with an unusually strong, high titer, antibody specific for platelet GPIIb/IIIa. Platelets from normal blood group O and HPA-1a (Pl^{A1}) positive donors were isolated, washed, and resuspended to a concentration of 1 x 10^9/ml. One ml of the platelet suspension was incubated with 1 mg ITP-prototype or normal IgG-F(ab´)$_2$ fragments. After washing and resuspension, the IgG-F(ab´)$_2$ blocked and unblocked platelets were used as a source of platelet antigens in the MAIPA assay.

VII. Lymphocyte preparation (paper III)

Mononuclear cells were prepared from heparin anticoagulated whole blood. The blood was diluted with an equal volume of 0.01 M PBS, pH 7.4, and 4 ml of the blood suspension were layered on 3 ml Ficoll-Isopaque (Pharmacia AB, Sweden) and centrifuged at 400 g for 30 min. The mononuclear cell fraction was harvested, washed twice, and finally resuspended in 0.01 M PBS.

VIII. DNA-preparation (paper III)

Genomic DNA was prepared from 5 ml EDTA anticoagulated whole blood using a miniscale salting out technique (Miller *et al,* 1988). The DNA concentration was measured at 260 nm using a spectrophotometer, and the samples were stored at -20°C until analysis.

IX. Immunoassays for platelet antibody detection

IX.1. Whole platelet ELISA (paper I). Briefly, blood group O platelets from normal donors were isolated from ACD-anticoagulated blood, washed with a Ringer-Citrate-Dextrose buffer, and 1 x 10^6 platelets were placed into each well of a tissue culture treated microtiter plate (Nunclon, NUNC, Denmark). After centrifugation, the plate was drained and blotted on absorbent paper. Following an overnight incubation in a vacuum dessicator the plates were washed twice and blocked with PBS/0.05%Tween-20/3%BSA for 90 min. Thereafter, 50 µl serum (diluted 1/10) was added in triplicate to the wells and incubated for 60 minutes at room temperature. Following washes, alkaline phosphatase conjugated goat anti-human Ig (IgG, A, M) was added and the plates were incubated for 45 minutes at room temperature. After four subsequent washes, the substrate p-nitrophenyl phosphate (PNPP) was added and the absorbance recorded at 405 nm using a microtiter plate reader. Four control sera were studied in association with each assay in order to serve as an internal standard. Reactions above the mean absorbance + 3SD recorded for these 4 control sera were regarded as positive.

IX.2 Modified antigen capture ELISA (MACE) (paper I). Washed normal platelets from blood group O donors were prepared, and 4 x 10^7 platelets were incubated (sensitized) with 100 µl test sera for 60 minutes, washed and solubilized in

TBS containing 1% Triton X-100. Insoluble material was removed by centrifugation and the supernatant sensitized platelet lysate was added in duplicate to the wells of a microtiter plate (Maxisorp, NUNC, Denmark), that had been coated with either MoAb AP1 (specific for platelet GPIb) or MoAb AP2 (specific for platelet GPIIb/IIIa). The IgG bound to the captured glycoprotein was detected using biotinylated MoAb HB43 (specific for the Fc-portion of human IgG) and an avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP kit, Vector Laboratories Inc., USA). PNPP was used as substrate and the absorbance was recorded at 405 nm in a microtiter plate reader. Four control sera were studied in association with each assay in order to serve as an internal standard. Reactions above the mean absorbance + 3SD recorded for these 4 control sera were regarded as positive. The MoAbs AP1 and AP2 were generous gifts from Drs Montgomery and Kunicki, at the Blood Research Institute, the Blood Center of SE Wisconsin, Milwaukee, USA.

IX.3 Flow cytometric detection of platelet antibodies (paper I). Platelet-rich plasma (PRP) was prepared from normal blood group O donors and the platelet count was adjusted to 500 x 10^9/l. One hundred µl of the platelet suspension was mixed with 100 µl of test serum and incubated for 1 hour at room temperature. After two subsequent washes the platelet bound immunoglobulins were detected by adding 100 µl fluorescein (FITC) conjugated goat anti-human immunoglobulin (secondary antibody). Three different secondary antibodies specific for human IgG (γ-chain), IgA (α-chain), and IgM (µ-chain) were employed. After 45 minutes incubation the platelets were washed twice, resuspended in 0.01 M PBS and immediately analyzed on the flow cytometer (FACScan, Becton Dickinson, USA). Four control sera were studied in association with each assay in order to serve as an internal standard. A median fluorescence intensity (MFI) above the mean MFI + 3SD recorded for these 4 controls was considered as positive.

IX.4 Antigen capture ELISA (paper V). Reactions of human anti-A and anti-A, B sera with platelet glycoproteins were studied using an antigen capture ELISA. In this assay, the glycoprotein of interest was immobilized by MoAbs attached to the wells of a microtiter plate. Briefly, 50 µl of the MoAbs CLB-IVC7 IgG (specific for platelet GPIV) or CLB-SW16 IgG (specific for GPV) (Chemicon AB, Sweden) in PBS, at a

concentration of 10 µg/ml, were added to each well of a 96-well microtiter plate (Maxisorp, NUNC, Denmark), that had been coated with affinity purified goat anti-mouse IgG, and incubated for 60 minutes at ambient temperature. After washing the plate was blocked for 30 minutes. The wells were then incubated with 50 µl diluted platelet lysate for 60 minutes, for capturing of the respective glycoprotein. Following four additional washes 50 µl of human anti-A or anti-A, B sera (Immucor Inc., USA) were added to the wells, and the plate was incubated for 60 minutes. Thereafter, the wells were washed four additional times, alkaline phosphatase conjugated goat anti-human Ig (IgG, A, and M specific) were added, and the plate incubated for another 45 minutes. Finally, the plate was washed four times and PNPP as substrate was added. The absorbance was recorded at 405 nm.

IX.5. Modified monoclonal antibody specific immobilization of platelet antigens (MAIPA) (paper II-IV). Washed platelets from normal blood group O donors were prepared. One hundred µl of the platelet suspension (1 x 10^8 platelets) were mixed (sensitized) with 100 µl test serum, incubated and thereafter solubilized in TBS containing 1% Triton X-100 and 100 µg/ml leupeptin. Insoluble material was removed by centrifugation. A MoAb, specific for the glycoprotein of interest, i.e. GPIb/IX and GPIIb/IIIa, was added to each well of a microtiter plate (Maxisorp, NUNC, Denmark) that had been coated with affinity purified goat anti-mouse IgG by overnight incubation. After washing the plate, 100 µl of "sensitized" platelet lysate were added to the wells in duplicate. After incubation and washes, 100 µl of either alkaline phosphatase conjugated goat anti-human IgG, goat anti-human kappa or goat anti-human lambda were added and the plate incubated. Finally, the plate was washed and PNPP as substrate was added. The absorbance was recorded at 405 nm using a microtiter plate reader. Four control sera were studied in association with each assay in order to serve as an internal standard. An absorbance greater than twice the mean absorbance recorded for the controls, or above control mean + 3SD was considered as a positive reaction.

X. Clonal excess analysis

X.1. Flow cytometric clonal excess analysis. Mononuclear cells were prepared from heparin anticoagulated blood by using a Ficoll-Isopaque gradient. One hundred µl of the mononuclear cell suspension, approximately 1 x 10^6 leukocytes, were mixed with rabbit serum to block non-specific binding sites. After washing the cells were divided into aliquotes and incubated with fluorochrome conjugated MoAbs or affinity purified polyclonal antisera (for details see paper IV); appropriate antisera were used for identification of T- (CD3+) and B-lymphocytes (CD19+), and for kappa- and lambda-immunophenotyping of CD19+ B-cells. After incubation the cells were washed twice, resuspended in 0.01 M PBS containing 1 % paraformaldehyde, and then analyzed on a flow cytometer (FACScan, Becton Dickinson, USA). The lymphocyte population was identified and a lymphocyte gate was set. The fluorescence of CD3-fluorescein (FITC) and CD19-phycoerytrin (PE) was analyzed to enumerate the number of T- and B-lymphocytes. A threshold was set at the nadir between CD19- and CD19+ cells in the kappa/lambda/control vs CD19 analysis, and a combined gate in FSC-SSC dot plot and Fl2 was constructed to include only CD19+ lymphocytes. The proportion of kappa- and lambda-expressing CD19+ lymphocytes was determined within this gate. A clonal excess of B-cells was considered to exist if either a discrete population with intermediate antigen expression was seen, or if a kappa/lambda ratio >3 or <0.5 was observed. Two patients with chronic lymphocytic leukemia, one having a kappa phenotype and the other a lambda phenotype, served as positive controls.

X.2. Polymerase chain reaction (PCR) amplification of the hypervariable complementarity determining region 3 (CDR3) of the heavy chain Ig-gene (paper III). This technique has previously been described by Brisco et al (1990). Briefly, consensus primers, flanking the hypervariable CDR3 region of the heavy chain Ig-gene, were employed, and the region of interest was amplified using a standard PCR-protocol with a "hot start" technique (for details see paper IV). The PCR products were separated on a 4.4 % NuSieve®/Agarose gel and visualized in UV-light after staining with ethidium bromide. DNA from healthy volunteers served as a negative control, and DNA from a patient with chronic lymphocytic leukemia, serially diluted in normal DNA, was used as a positive control.

XI. Immunoprecipitation (paper V)

One hundred µl aliquotes of biotinylated or unlabelled platelet suspensions, at a concentration of 1 x 10^9/ml, were each incubated with the MoAbs CLB-IVC7 (anti-GPIV) or CLB-SW16 (anti-GPV) for 60 minutes. The antibody-sensitized platelets were then washed four times with PBS/EDTA, and solubilized in 100 µl TBS, pH 8.5, containing 1% Triton X-100, leupeptin, and phenylmethylsulfonyl fluoride (PMSF). The insoluble material was removed by centrifugation, and 20 µl of a 1:1 slurry of Protein A-Sepharose CL-4B (Pharmacia AB, Sweden) were added to the supernatant, and the mixture was incubated for 3 hours at 4° C. The Protein A beads were pelleted and washed four times. Bound proteins were eluted by resuspending the final bead pellet in 70 µl of SDS-PAGE sample buffer and incubating the mixture at 100° C for 5 minutes. The mixture was then centrifuged at 12,000 g for 1 min. The supernatants were electrophoresed on a 5 to 15% gradient SDS-PAGE gel under nonreduced and reduced conditions using the discontinuous buffer system of Laemmli (1970). Broad range biotinylated weight standards (Bio-Rad Lab., USA) were included to allow localization of the major labeled platelet proteins. After completion of the run the gel was equilibrated in Towbin buffer (Towbin *et al,* 1979) for 15 minutes, and then electrotransferred to a 0.45 µm pore-size nitrocellulose membrane (Bio-Rad Lab., USA) using the same buffer. The membrane was then blocked with TBS-0.05%Tween-3%BSA. After two subsequent washes the lanes of the membrane holding immunoprecipitated biotinylated platelet GPs were cut out and incubated with an avidin-biotin-alkaline phosphatase complex diluted 1:500 in TBS-Tween-BSA. After an additional four washes in TBS-Tween the bands were developed with the NBT/BCIP substrate. The remaining lanes of the membrane, holding immunoprecipitated unlabeled platelet GPs were used for immunoblotting.

XII. Immunoblotting of the immunoprecipitated platelet GPs (paper V)

The membrane lanes holding immunoprecipitated and unlabeled platelet GPs were incubated with the MoAb 402-3D9 (specific for blood group A antigen) (New Monocarb, Sweden) for 2 hours at 4° C. The membrane was then washed and incubated with biotinylated goat anti-mouse IgG for 60 minutes. After additional washings the bands were developed using the ABC-AP kit and NBT/BCIP as substrate, as described above.

RESULTS AND DISCUSSION

Comparison of three methods for detection of platelet antibodies in patients with chronic ITP (paper I)

Sera from an unselected group of patients with chronic ITP were used for this study, and the purpose was to compare different techniques suitable for large scale screening of serum platelet antibodies. Two of these methods used whole platelets as targets for the antibodies (flow cytometry and whole platelet ELISA), and the third assay was an antigen-specific ELISA (MACE), in which GPIb/IX and GPIIb/IIIa were captured with monoclonal antibodies. The whole platelet based assays will irrespective of their specificity detect all immunoglobulins attached to the platelet surface. Thus, these techniques cannot discriminate between platelet autoantibodies bound to membrane GPs, alloantibodies bound to HLA-antigens, and immune-complexes bound to the platelet FcγRII-receptors.

We detected platelet antibodies in 35% of the ITP-sera using the whole platelet ELISA, and in about 45% of the sera with the flow cytometric technique. This frequency of serum platelet antibodies in ITP is in accordance with the reports of other investigators, in which similar techniques were employed, i.e. the platelet membrane ELISA (Winiarski & Ekelund, 1986) and the platelet suspension immunofluorescence test (PSIFT) (von dem Borne *et al,* 1980). The present immunoglobulin class analysis of platelet antibodies demonstrated that IgG was the most frequent antiplatelet immunoglobulin (26/29 patients); however, IgG was sometimes detected together with IgA and/or IgM. Only three sera displayed platelet antibodies only belonging to the IgA and/or IgM class, a finding in accordance with the results of von dem Borne et al (1980).

By employing an ELISA (MACE), which detected antibodies binding to platelet GPIb/IX and GPIIb/IIIa, we found that 23 out of 65 ITP sera (35%) possessed antibodies with these specificities; antibodies against GPIb/IX were most frequently observed, either alone or in combination with anti-GPIIb/IIIa. Of the patients positive in the MACE-assay only 61% gave positive results in the whole platelet ELISA and 78% in the flow cytometric assay. In previous reports the finding of autoantibodies

against GPIb/IX has been considered uncommon (Woods *et al,* 1984a; Szatkowski *et al,* 1986; McMillan *et al,* 1987). However, by employing the MAIPA-assay, a technique similar to the antigen-specific assay used by us, Kiefel et al (1991) found that antibodies against GPIb/IX were as common as antibodies against GPIIb/IIIa. None of the assays employed in the present study had a sensitivity high enough to be recommended for routine diagnostic use in chronic ITP. However, in certain cases of ITP and for thorough evaluation of the autoimmune process in ITP, it seems reasonable to use a whole platelet based assay in conjunction with an antigen specific assay; the whole platelet based assay might detect uncommon antibody targets, and the specificity of the antibodies can be confirmed with the antigen specific ELISA by using appropriate capturing MoAb.

Light chain restricted autoantibodies in chronic ITP (paper II)

Assuming that the autoantibody production in chronic ITP is due to an expansion of one or a few autoreactive B-cell clones, and if the autoantigen repertoire on platelet glycoproteins is limited, the antibodies should possess monoclonal characteristics, e.g. a restriction as to their light chain phenotype and subclass. Indeed, in the present study we found that the GPIb/IX and GPIIb/IIIa specific antibodies in ITP frequently were restricted to either kappa or lambda light chains, supporting the hypothesis of clonally derived autoantibodies in ITP.

Several additional lines of evidence support the hypothesis of a limited autoantigen repertoire in chronic ITP: first, certain GP-specific murine monoclonal antibodies compete with the ITP autoantibodies in their binding to the glycoprotein (Varon & Karpatkin, 1983); second, human monoclonal autoantibodies generated from a patient with ITP behaved as if they were selected by an antigen-driven clonal maturation (Nugent *et al,* 1989; Hiraiwa *et al,* 1990); and third, proteolytic fragments of GPIIIa are frequent targets for autoantibodies in ITP (Kekomaki *et al,* 1991).

Further support for the hypothesis of clonal autoantibodies in chronic ITP has been obtained by two independent investigators; on a rather crude patient material with immune thrombocytopenias they found light chain restricted platelet antibodies, and expanded clonal B-cell populations were commonly present (van der Harst *et al,* 1990;

Christie *et al,* 1993). In the present study we employed sera from 19 patients with an unequivocal diagnosis of chronic ITP and secondary causes, e.g. lymphoproliferative disorders, were specifically ruled out. All of our ITP patients were also known to have serum antibodies binding to GPIIb/IIIa and/or GPIb/IX. Thus, the novel finding of the present work was that chronic ITP patients without any secondary cause displayed light chain restricted platelet antibodies in a high frequency.

Light chain restricted autoantibodies but no circulating clonal B-lymphocytes (paper III)

The homogeneity of Ig-proteins can be used as a clonality marker in B-cell disorders, e.g. the presence of a monoclonal gammopathy, a disproportional number of B-cells having either a kappa (κ) or lambda (λ) light chain immunophenotype, and clonal rearrangements of hypervariable Ig-gene regions. The flow cytometric clonal excess analysis is based on the fact that the normal ratio of κ- and λ-expressing B-lymphocytes is approximately 3:2 (Johnson & Olofsson, 1993). Thus, if all B-cells in a lesion express either the κ or the λ immunophenotype, it is very likely that the lesion is a clonally derived tumor. On the other hand, if the ratio of κ- and λ-expressing B-cells is fairly normal it is suggestive that the lesion is a polyclonal or non-neoplastic proliferation (Abbas, 1994).

Furthermore, it is now well established that rearrangements of separate Ig gene segments provide the diversity for antigen recognition by B-cells. These events can provide useful markers of clonality, cellular origin, and stage of B-cell differentiation in the study and diagnosis of B-cell disorders. In particular, the diversification of heavy chain genes is generated by recombination of variable (V_H), diversity (D) and joining (J_H) gene segments. Additional variability in the heavy chain gene sequence is introduced by random deletions and insertions of nucleotides at the V-D and D-J junctions, as well as somatic mutations (Tonegawa, 1983). The junctional sequences correspond to the complementarity determining region 3 (CDR3) of the heavy chain gene and are quite specific to individual alleles. Recently, a number of laboratories have described application of polymerase chain reaction (PCR) techniques to significantly increase the sensitivity and specificity of clonal B-cell detection (Brisco

et al, 1990; Trainor et al, 1990; Billadeau et al, 1991). Using primers homologous to consensus sequences in the V_H and J_H gene segments, the CDR3 region can be amplified, sequenced, and subsequently used as a probe to detect residual malignant B-cells clones from as few as 1 in a background of 10^5 cells (Trainor et al, 1990).

In the present study (paper III) we were able to reconfirm our previous finding (paper II) of light chain restricted platelet antibodies in chronic ITP, by analyzing the platelet antibody κ/λ-phenotype in 20 new patients. However, in these patients we could not detect any clonally expanded B-cell population, by employing a flow cytometric "clonal excess" analysis or by PCR rearrangement studies of the heavy chain Ig-gene. These latter results are consequently at variance with the reports by van den Harst et al (1990) and Christie et al (1993). Indeed, there are several possible explanations for the contradictory findings. First, van der Harst et al (1990) studied 11 patients with ITP, 4 of whom even displayed paraproteinemia and only four were diagnosed as chronic ITP. Further, no data characterizing platelet reactive antibodies were presented in their report. Also, the study by Christie et al (1993) was performed on a crude patient material with immune thrombocytopenias and miscellaneous malignant hematological disorders, holding only two patients with chronic ITP; the majority of the molecular studies were performed on blood samples obtained up to 24 months after the serological studies, and a discrepancy was found between the light chain phenotypes of the platelet reactive antibodies and the clonal B-cells. Indeed, different mechanisms might account for the presence of clonal B-cells in patients with thrombocytopenia due to autoimmunity, alloimmunity, and drug-induced-immunity, respectively. The chronic ITP patients exploited in our study were not confounded by other disorders which might affect the results obtained from clonality analysis. Thus, patients afflicted with secondary immune thrombocytopenias and particularly paraproteinemias were excluded from the present study. Second, the flow cytometric clonal excess assay and Ig-gene rearrangement analysis by PCR, at their best have detection limits of approximately 10% and 1%, respectively (Trainor et al, 1990; Agrawal et al, 1992; Johnson & Olofsson, 1993). Hence, clonally expanded B-cell populations, if they really exist in chronic ITP, are most probably below the detection limits of the assays employed.

Glycoprotein IIb/IIIa autoantigenic repertoire in ITP (paper IV)

The objective of this paper was to further disclose the autoantigenic repertoire carried by the GPIIb/IIIa complex. IgG-F(ab´)$_2$ fragments prepared from two prototype ITP sera, known to possess a high titer GPIIb/IIIa specific antibody, were each able to block the binding of serum GPIIb/IIIa-specific antibodies in 6 (55%) and 7 (64%) out of 11 patients with chronic ITP, respectively. The blocking of serum IgG binding to GPIIb/IIIa could be due to the fact that prototype IgG-F(ab´)$_2$ fragments preoccupied the homogenous autoepitopes. Another explanation could be that autoantigenic determinants on GPIIb/IIIa were perturbed by the binding of prototype IgG-F(ab´)$_2$ fragments to a very close determinant. Conversely, failure in blocking the binding of serum IgG to GPIIb/IIIa can be a consequence of: first, that the prototype IgG-F(ab´)$_2$ fragments and autoantibodies bind to heterogenous epitopes on GPIIb/IIIa; second, that in extremely rare circumstances, the GPIIb/IIIa reactive antibodies in ITP sera could be platelet specific alloantibodies beyond the HPA-1 ($PI^{A1/A2}$) system.

Furthermore, our data are in accordance with the concept of a homogenous autoantibody repertoire, specific for GPIIb/IIIa, in a significant fraction of chronic ITP patients (Varon & Karpatkin, 1983; Fujisawa *et al,* 1990; Kekomaki *et al,* 1991). By analyzing the competitive binding between human autoantibodies and murine MoAb specific for GPIIb/IIIa Fujisawa et al (1990) observed a decreased amount of platelet-bound anti-GPIIb/IIIa MoAb in 5 out of 22 (23%) patients with chronic ITP, indicating binding of autoantibodies directed toward antigenic determinants on or close to the epitope of the MoAb. Using enzyme cleaved GPIIIa fragments, Kekomaki et al (1991) found that a 33 kD chymotryptic core fragment of GPIIIa was bound by 48% (15/31) of patients with chronic ITP. Furthermore, employing the hydrophatic complementarity approach, De Souza et al (1992) reported that 5 out of 17 (30%) GPIIb/IIIa reactive ITP sera also bound to a peptide deduced from the complementary nucleotide sequence to that which codes for the Arg - Gly - Asp (RGD) domain in fibronectin. Recently, Fujisawa et al (1992) showed that 13 out of 21 (62%) GPIIb/IIIa reactive sera bound primarily to the carboxy terminus of GPIIIa.

In summary, we have provided evidence that as defined by serum autoantibodies, half of the ITP patients with GPIIb/IIIa reactivity seem to share a homogenous GPIIb/IIIa

autoantigenic repertoire. Considering that light chain restricted antibodies and circulating clonal B-cells have been shown to exist in chronic ITP (van der Harst *et al,* 1990; Christie *et al,* 1993), the total number of autoantigenic epitopes on GPIIb/IIIa ought to be quite limited.

Evidence for an expression of blood group A antigen on platelet GPIV and GPV (paper V)

The expression of blood group ABH determinants on platelet has been assigned to glycolipids and to glycoproteins Ib, IIa, IIb, IIIa, and PECAM (Santoso *et al,* 1991; Santoso *et al,* 1993; Ogasawara *et al,* 1993; Hou *et al,* 1996). Most recently we observed that a MoAb specific for blood group A antigen immunoblotted some other proteins derived from SDS-PAGE separated blood group A_1 platelet lysates (Hou *et al*, 1996). These ABO-expressing platelet proteins had molecular weights ranging between 70-90 kD, and their electrophoretic mobility was unaffected by 2-mercaptoethanol reduction (Hou *et al*, 1996). Considering the molecular weight and the number of potential glycosylation sites on platelet GPIV and GPV (Oquendo *et al,* 1989; Legrand *et al,* 1991; Modderman *et al,* 1992; Lanza *et al,* 1993), we found it theoretically suggestive that these two glycoproteins should contain ABH blood group determinants, and could account for the 70-90 kD uncharacterized bands seen in the anti-A immunoblot.

The following lines of evidence were obtained by us, regarding the characterization of the 70-90kD platelet proteins holding the blood group A antigen. First, by antigen capture ELISA, wherein the MoAbs CLB-IVC7 (specific for GPIV) and CLB-SW16 (specific for GPV) were used to hold the corresponding antigens, human anti-A specifically bound to these proteins derived from A_1-platelets. Neither GPIV nor GPV derived from A_2-, B-, or O-platelets bound anti-A. In the same assay, human anti-A, B bound to both GPIV and GPV derived from A_1 as well as B-platelets. This latter finding suggests that blood group B-antigen is also expressed on GPIV and GPV derived from blood group B platelets. Second, in a Western blot assay using immunoprecipitated GPIV and GPV as antigens, MoAb anti-A immunostained GPIV and GPV precipitated from A_1, but not from A_2 and O platelets. These results demonstrate that blood group A antigen is expressed on platelet GPIV and GPV.

It is noteworthy that binding of human polyclonal anti-A in the ELISA assay and that of murine MoAb anti-A in the immunoblot assay, were only detected by platelets from blood group A_1 individuals, whereas A_2 platelets did not display any significant reactivity. Similar findings have been observed in the study of other ABO-expressing platelet glycoproteins (Holgersson *et al,* 1990; Santoso *et al,* 1991; Hou *et al,* 1996).

The present observations provide additional biochemical basis for the clinical refractoriness in the transfusion of ABO-incompatible platelets, (Aster, 1965, Duquesnoy *et al,* 1979; Brand *et al,* 1986; Heal *et al,* 1987; Ogasawara *et al,* 1993). Also, a platelet A_1/A_2 difference was evidenced, which might explain why blood group A_2 platelets serologically behave as blood group O platelets in clinical platelet transfusions (Skogen *et al,* 1988).

CONCLUSIONS

I. Whole platelet based techniques and antigen specific techniques used for platelet antibody detection have their merits and demerits; it appears reasonable that they should be used together in evaluation of the autoimmune process of chronic ITP.

II. At least half of the ITP patients with GPIIb/IIIa reactivity seem to share a homogenous autoantigenic repertoire. Considering the evidences for clonal autoantibodies in ITP, it is justifiable to assume that the number of autoantigenic epitopes on platelet GPIIb/IIIa is quite limited. If this hypothesis holds true, novel diagnostic and treatment strategies can be opened, e.g. anti-idiotypic therapies and specific immunabsorption.

III. Blood group A-antigen is expressed on platelet GPIV and GPV derived from blood group A_1 but not A_2 platelet. Hereby, additional biochemical basis for the clinical refractoriness in the transfusion of ABO-incompatible platelets is provided. Also, this finding emphasizes that even in glycoprotein specific immunoassays should proteins from blood group O platelets be used as the antibody target, in order to avoid interference from ABO-antibodies.

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