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INVESTIGATIONS OF PLATELET IgG IN IMMUNE AND NON-IMMUNE THROMBOCYTOPENIA

By

MARY HUGHES, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

DOCTOR OF PHILOSOPHY

McMaster University

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PLATELET IgG IN IMMUNE AND NON-IMMUNE THROMBOCYTOPENIA

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AUTHOR: Mary Hughes, B.Sc. (University of Western Ontario), M.Sc. (McMaster University)

SUPERVISOR: Dr. John G. Kelton, M.D., Professor, Medicine

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ABSTRACT

Idiopathic thrombocytopenic purpura (ITP) is a common autoimmune disorder characterized by the premature destruction of autoantibody-sensitized platelets. For many years, elevated platelet-associated IgG (PAIgG) was thought to be a diagnostic characteristic of ITP. However, observations that PAIgG is increased in both immune and non-immune thrombocytopenia, questioned the clinical significance of PAIgG measurements, and the mechanism(s) by which IgG accumulates in platelets. The origin of IgG on the surface of platelets of patients with non-immune thrombocytopenia, is not known. Additionally, it is not clear why these IgG-sensitized platelets escape destruction by phagocytic cells of the reticuloendothelial system. In this investigation, possible biological explanations for the elevated PAIgG in adult patients with ITP and non-immune thrombocytopenia, were examined. Traditionally, laboratory investigation of ITP, has focused on the development of serologic assays to measure anti-platelet autoantibodies. As an alternative investigative approach, ultrastructural techniques were used to evaluate platelets from patients with ITP and non-immune thrombocytopenia. Using these techniques, it was possible to localize IgG in platelets, and determine the immunomorphologic characteristics of PAIgG in patients with immune and non-immune thrombocytopenia. The results of these investigations demonstrated, that apart from differences in numbers, ITP platelets and platelets from patients with non-immune thrombocytopenia are morphologically identical to normal platelets and show no evidence of structural abnormalities. Additionally, elevated total PAIgG levels in thrombocytopenic patients are not attributable to alterations in the physical characteristics of platelets, including increased platelet size or increased numbers of storage granules per platelet, but reflected quantitative abnormalities in the pool of exogenous α-granule proteins. Immunomorphologic characteristics of PAIgG in patients with ITP

and non-immune thrombocytopenia differ. In patients with ITP, elevated PAIgG is observed both within and on the surface of platelets. In patients with non-immune thrombocytopenia, elevated PAIgG was observed within but not on the platelet surface. In these platelets, IgG is not elevated on the platelet surface and, consequently, platelets were not prematurely cleared by phagocytic cells of the reticuloendothelial system. These results further explain why patients with non-immune thrombocytopenia have a normal platelet lifespan. Demonstrations that immunomorphologic characteristics of surface PAIgG, in platelets from patients with non-immune thrombocytopenia change over time suggests that *in vitro* leakage of α-granule IgG onto the surface of platelets may account for the origin of surface PAIgG in these patients. Immunomorphologic characteristics of surface PAIgG in patients with immune and non-immune thrombocytopenia suggest that rim patterns of surface PAIgG in ITP reflect the binding of autoantibodies to glycoprotein targets on the platelet surface whereas, "beaded necklace"patterns of surface PAIgG in non-immune thrombocytopenia reflect IgG exocytosis from internal platelet storage pools *in vitro*. In summary, ultrastructural techniques have been useful for the study of early structural events leading to the pathogenesis of disease and more recently have been utilized in studies of platelet pathology. Whereas current measurements of PAIgG do not provide good diagnostic or prognostic value, ultrastructural patterns of IgG distribution in ITP platelets may in themselves, or in conjunction with other laboratory measures, provide additional information about a common thrombocytopenic disorder.

For my parents, Bill and Alice Hughes

my sister, Denise

and for Steve, the extraordinary person I share my life with.

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LIST OF SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used within this thesis are defined below.

β-TG	beta-thromboglobulin
BSA	bovine serum albumin
EDTA	ethylenediamine-tetraacetic acid
g	acceleration due to gravity
hIgG	human immunoglobulin G
ITP	idiopathic thrombocytopenic purpura
kDa	kiloDalton(s)
OD	optical density
PAIgG	platelet-associated IgG
PF4	platelet factor 4
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PRP	platelet-rich plasma
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER 1

Background information and introduction

1.1 Foreward

Idiopathic thrombocytopenic purpura (ITP) is a common autoimmune disorder and a frequent cause of thrombocytopenia (Simpson *et al*, 1988). Since the original description of "morbus maculosus haemorrhagicus" by Werlhof in the eighteenth century (Jones and Tocantins, 1933), the pathophysiology of thrombocytopenia in ITP has been the subject of study for many years. In 1951, Harrington transfused plasma from patients with ITP into healthy volunteers and demonstrated that a factor present in the plasma of ITP patients, could provoke a profound thrombocytopenia in normal recipients (Harrington *et al*, 1951). In 1965, Shulman demonstrated that the thrombocytopenic factor was an IgG anti-platelet antibody that reacted with both autologous and homologous platelets, and could be removed by adsorption with platelets (Shulman *et al*, 1965a, b). In 1969, Aster and Keene demonstrated that the spleen and liver played major roles in the clearance of antibody-sensitized platelets in ITP (Aster and Keene, 1969), with later studies showing that it was the phagocytic cells of the spleen which were predominantly responsible for platelet clearance (Leddy and Swisher, 1975; Anderson and Looney, 1986; Unkeless, 1989; Schreiber *et al*, 1989). Finally, in 1975, Dixon demonstrated that patients with ITP had abnormally high concentrations of platelet-associated IgG (PAIgG). This observation and the demonstration that ITP was caused by anti-platelet autoantibodies led to an implicit assumption that platelet IgG was anti-platelet antibody and that elevated PAIgG levels were a diagnostic characteristic of ITP (George, 1990). Since the initial detection of gammaglobulin in washed human platelets by Salmon in 1958, over 40 years of PAIgG investigations have led to two generally accepted observations. Firstly, platelets from patients with ITP have elevated levels of PAIgG (Dixon *et al*, 1975; Luiken *et al*, 1977; Cines and Schreiber,

1979; Kelton and Steeves, 1983; George *et al*, 1985; George and Saucerman, 1988). Secondly, elevated PAIgG is not specific for ITP and is observed in most thrombocytopenic disorders including disorders which are not thought to be immune-mediated such as aplastic anemia and leukemia (George and Saucerman, 1988; Kelton *et al*, 1989; Heaton *et al*, 1988; George, 1989; Mueller-Eckhardt *et al*, 1980).

1.2 Idiopathic thrombocytopenic purpura (ITP)

1.2.1 Introduction

ITP is an autoimmune disorder defined by a low platelet count (less than 150 x 10^9/L) in the absence of other clinically-apparent causes of thrombocytopenia (McMillan, 1981). This disorder has been estimated to affect approximately 7 individuals per 100,000 annually in North America and is characterized by premature platelet destruction through removal of antibody-sensitized platelets by phagocytic cells of the reticuloendothelial system (George *et al*, 1996a; McMillan, 1981). Despite some 40 years of investigation, there is no diagnostic test for ITP. The physical examination of patients with ITP is usually normal with the exception of clinical manifestations of thrombocytopenia, such as frequent bruising, bleeding from the mucus membrane and menorrhagia in women (Bussel and Schreiber, 1991). In some patients, clinical signs of bleeding are absent and thrombocytopenia is detected during a routine physical examination. Examination of the bone marrow indicates a normal or increased number of megakaryocytes, with no abnormalities of other hematopoietic cells, apart from concurrent iron deficiency anemia (Bussel and Schreiber, 1991). Intracranial, retinal, or abdominal hemorrhage is rare (Bussel and Schreiber, 1991; George *et al*, 1996b) and deaths from hemorrhage occur in only 5% of adults with chronic ITP (Scaradavou and Bussel, 1998) and in less than 1% of children (George *et al*, 1996b).

1.2.2 Childhood ITP (acute) versus adult ITP (chronic)

ITP can occur in both children and adults and can be either acute (duration of disease <6 months) or chronic (>6 months) (Blanchette *et al*, 1998). Characteristically, childhood ITP is an acute, self-limiting illness that occurs in previously healthy children following an infectious bacterial or viral illness (Blanchette *et al*, 1998). The peak age of occurrence is between ages two and four years, with both males and females affected equally (George and Raskob, 1998). This form of ITP is generally benign and resolves spontaneously in up to 90% of children within several weeks or months, often without treatment (Lightsey 1980; George *et al*, 1996a; George and Raskob, 1998).

In comparison, adult ITP is typically insidious in onset, chronic in nature, and predominant in young women (George and Raskob, 1998). Chronic ITP may occur as an isolated condition or less frequently in association with other disorders, including systemic lupus erythematosus, chronic lymphocytic leukemia and Hodgkin's disease (Karpatkin and Siskind, 1969; Ebbe *et al*, 1962). In the majority of adults the incidence of spontaneous recovery is less than 20%, with most patients requiring long-term treatment strategies (George *et al*, 1996a). ITP is most common in women of child-bearing age and is the third most common cause of thrombocytopenia in pregnancy (Burrows and Kelton, 1990a, b). Since IgG anti-platelet autoantibodies can cross the placenta there is also the risk of thrombocytopenia occurring in the neonate (Burrows and Kelton, 1990a, b). Currently, there is no way to predict with certainty which baby born to a mother with ITP will be severely thrombocytopenic and which will be healthy (Burrows and Kelton, 1990a, b).

1.2.3 Pathophysiology

In patients with ITP, platelet surface membrane proteins become antigenic for unknown reasons (McMillan, 2000). This change in antigenicity, results in stimulation of the immune system and autoantibody production (McMillan, 2000). The binding of autoantibodies to platelets, leads to their removal from the circulation, either by phagocytosis or complement-induced lysis (McMillan, 2000). Autoantibodies may also bind to megakaryocytes and affect thrombopoiesis, resulting in reduced platelet production (Heyns *et al*, 1986; Ballem *et al*, 1987). In general, autoantibody-induced platelet destruction in ITP, is mediated by two factors. Firstly, the production of IgG anti-platelet autoantibody and, secondly the functional capacity of the reticuloendothelial system.

1.2.3.1 Autoantibodies in ITP

1.2.3.1.1 Evidence of IgG autoantibodies in ITP

The first evidence that ITP was an autoimmune disease, came from *in vivo* transfusion studies. In these classic experiments, infusions of ITP patient plasma into healthy volunteers or patients with inoperable malignant neoplasms, produced a marked and transient thrombocytopenia in more than one-half of the recipients (Harrington *et al*, 1951). Subsequent studies showed that the thrombocytopenic effect was dose-dependent and less severe in splenectomized patients and in patients treated with corticosteroids (Shulman *et al*, 1965). These observations, together with reports that mothers with ITP often gave birth to children who developed a transient thrombocytopenia suggested the transfer of a humoral factor was causative (Robson and Davidson, 1950; Epstein *et al*, 1950). Initial laboratory investigation of the thrombocytopenic factor in ITP patients demonstrated that it co-purified in the IgG fraction of serum, reacted with both homologous and autologous platelets, and could be removed by adsorption with platelets (Harrington *et al*, 1951; Shulman *et al*,

1965). Further analysis showed that it had the characteristics of an IgG immunoglobulin in that it was stable at 56°C, had a similar molecular weight, could be passively transferred across the placenta, and that its activity could be neutralized with a globulin fraction of rabbit anti-human IgG antisera, but not anti-IgM, anti-IgA, anti-IgD, or normal rabbit globulin (Epstein *et al*, 1950; Karpatkin and Siskind, 1969; Karpatkin *et al*, 1972a). Collectively, these observations suggested that the thrombocytopenia in ITP was caused by the production of an IgG anti-platelet autoantibody. Further support for an autoimmune mechanism for ITP, came from studies demonstrating that splenic lymphocytes from patients with ITP, when cultured *in vitro*, produced IgG antibodies which bound to both autologous and homologous platelets (McMillan *et al*, 1974b) as well as megakaryocytes (McMillan *et al*, 1978). In these studies, $F(ab')_2$ fragments of ITP splenic IgG and ITP platelet IgG retained the ability to bind to normal platelets (McMillan *et al*, 1972, 1980; Karpatkin *et al*, 1972b). These investigations provided conclusive evidence that ITP was caused by anti-platelet IgG autoantibodies which bound to platelets in an antigen-dependent fashion and could be produced by splenic lymphocytes in culture.

1.2.3.1.2 Platelet proteins as autoantibody targets in ITP

In 1982, van Leeuwen demonstrated that antibody eluates from ITP platelets bound to normal platelets, but not to thrombasthenic platelets deficient in the membrane glycoprotein complex IIb/IIIa. These initial studies suggested that platelet membrane glycoproteins may be important autoantibody targets in ITP. Subsequently, with the development of antigen-specific assays, autoantibodies reactive with one or more membrane glycoprotein can be detected in 50% to 80% of ITP patients (Berchtold *et al*, 1993; Fujisawa *et al*, 1993; He *et al*, 1994; Warner *et al*, 1999). Of these detectable autoantibodies, approximately 75% are directed against the glycoprotein IIb/IIIa (GPIIb/IIIa), or

glycoprotein Ib/IX (GPIb/IX), complexes (McMillan *et al,* 1987; Kiefel *et al*, 1991). In the other 25%, it is thought that other membrane glycoproteins are involved (McMillan, 2000). Some of these less frequent antigenic targets include, GPIa/IIa, GPIV (He *et al*, 1994; Pfueller *et al*, 1990), GPVI (Sugiyama *et al*, 1987), and GPV (Wadenvik *et al*, 1998).

Localization of the antigenic epitopes on membrane glycoproteins has only recently been investigated (McMillan, 2000). These studies have been limited primarily to investigation of the most common platelet autoantigen GPIIb/IIIa. Using enzyme-cleaved GPIIb or GPIIIa fragments and synthetic GPIIIa peptides, many plasma anti-GPIIb/IIIa autoantibodies have been shown to react with normally concealed cytoplasmic epitopes while platelet-eluted anti-GPIIb/IIIa autoantibodies have been shown to react with epitopes on the extracellular domains (Fujisawa *et al*, 1991, 1993; Kosugi *et al*, 1996). It is unclear why plasma autoantibodies react with antigenic domains normally not thought to be exposed on the platelet surface. One proposed explanation is that cytoplasmic antigens may be exposed during the course of platelet destruction resulting in autoantibody formation (Fujisawa *et al*, 1991). It remains to be determined what role, if any, this type of plasma autoantibody plays in the pathogenesis of ITP and other autoimmune disorders. More recently, Fujisawa *et al* (1993) have demonstrated that platelet-eluted anti-GPIIb/IIIa autoantibodies in patients with chronic ITP bind to cation-dependent epitopes. Whether these epitopes depend on a conformationally intact glycoprotein complex or are located near the calcium-binding sites on either GPIIb or GPIIIa has yet to be determined (Fujisawa *et al*, 1993).

1.2.3.1.3 Investigations of target antigens in different forms of ITP

Acute ITP is most often observed in children and is predominantly benign and self-limiting. In comparison, chronic ITP or adult ITP has a more insidious presentation and poses more severe management problems. These two forms of ITP are thought to be initiated by different pathological mechanisms. In acute ITP, the production of anti-platelet autoantibodies is thought to be linked to a transient antiviral immune response (Winiarski, 1998). In this way, the mechanism of platelet autoantibody production may occur as a response to cell injury or exposed cryptoantigens or through molecular mimicry and formation of anti-idiotypic antibodies (Haffler and Flavell, 1996; Vaughan *et al*, 1995). In comparison, the mechanism of platelet autoantibody production in chronic ITP is thought to involve alterations in immunoregulation such as imperfect thymic deletion of self-reactive T cells and/or clonal expansion of autoantibody producing B cells (McMillan, 2000).

Early investigations of the target antigen of serum autoantibodies in patients with acute and chronic ITP suggested that different platelet glycoproteins were important targets for different forms of ITP. In these studies, serum antibodies in children with acute ITP recognized an 80,000 Dalton platelet protein thought to represent GPV (Beardsley *et al*, 1985; Stricker *et al*, 1986). Other studies indicated that serum antibodies recognized GPIIIa in chronic ITP but not in acute ITP (Beardsley *et al*, 1984). More recently, antigen-specific assays have demonstrated that platelet-specific IgG autoantibodies can be detected in both acute and chronic ITP (Berchtold *et al*, 1989; Taub *et al*, 1995). In these studies, GPIIb/IIIa is the predominant platelet target for both forms of ITP, with IgG autoantibodies being detected in a lower proportion of acute versus chronic ITP cases (Berchtold *et al*, 1989; Taub *et al*, 1995).

1.2.3.2 Functional capacity of the reticuloendothelial system

In addition to the presence of an autoantibody, the pathophysiology of ITP is mediated by the functional capacity of the reticuloendothelial system (Kelton, 1987). In ITP, the binding of autoantibodies to platelets, results in their clearance from the circulation by macrophages through Fc binding (McMillan *et al*, 1974a). This process of immune phagocytosis occurs primarily in the spleen and, to a lesser extent, in the liver and bone marrow.

1.2.3.2.1 Primary sites of platelet destruction in ITP

In ITP, the spleen is the primary site of destruction of autoantibody-sensitized platelets (Anderson and Kelton, 1990). The unique anatomy of the spleen allows it to function as a filter of the peripheral blood, removing bacteria, foreign material and antibody-coated cells (Weiss and Tavassoli, 1970; Neiman and Orazi, 1999). The spleen is also a secondary lymphoid organ and plays an important role in immune surveillance and response (Chapman and Newman, 1999; Weiss, 1995). The spleen has no afferent lymphatics (Weiss, 1995). Blood entering the spleen must pass through a complex microcirculation in order to exit the spleen, and rejoin the circulation (Weiss, 1995). This tortuous journey, results in prolonged exposure of antigens to antigen-processing cells in the marginal zone of the white pulp, and macrophages in the red pulp (van Krieken and te Velde, 1988). In ITP, the close proximity of self-reactive splenic B and T cells to the circulating blood allows for steady exposure of platelet antigens to the immune system and the production of large amounts of anti-platelet autoantibody (McMillan *et al*, 1972, 1974b; Karpatkin *et al*, 1972b; Chadburn, 2000). Since approximately 35% of the platelet mass is present in the spleen at all times, these autoantibodies can immediately bind to their platelet-specific antigens resulting in the clearance of platelets by cordal macrophages, via Fc binding (Chadburn, 2000). In this way, the spleen is both an important site of

autoantibody production and platelet destruction. Currently, splenectomy remains the most definitive treatment for patients with ITP.

Additional sites of platelet destruction in ITP, include the liver and bone marrow. The liver, in comparison to the spleen, contains no intra-organ platelet pool, produces no anti-platelet autoantibodies, and has a more rapid circulation (McMillan, 2000). In cases of severe ITP, in which platelets are heavily sensitized with autoantibody or in patients who relapse following splenectomy, the liver becomes the predominant site of platelet destruction (Aster and Jandl, 1964; Crome and Mollison, 1964; Jandl and Kaplan, 1960; McMillan *et al*, 1972). Finally, the bone marrow is also a site of platelet destruction. The bone marrow contains a reticuloendothelial system and is capable of producing anti-platelet autoantibody (McMillan, 2000). Since anti-platelet autoantibody can bind to megakaryocytes, as well as platelets, it is thought that inhibition of platelet production as well as premature platelet destruction contributes to the thrombocytopenia in some patients (McMillan, 2000).

1.2.3.2.2 Factors influencing the Fc-dependent function of the reticuloendothelial system

There are several factors which influence the Fc-dependent function of the reticuloendothelial system. These factors include: (1) Fc receptor expression, (2) IgG subclass, (3) the plasma concentration of monomeric IgG, and (4) the amount of IgG and complement on the cell surface (Anderson and Kelton, 1990).

1.2.3.2.2.1 Fc receptor expression

Three distinct classes of Fc receptors, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), are specific for the IgG class of immunoglobulins (Gessner *et al*, 1998). These receptors are found on macrophages and monocytes and serve to link IgG antibody-mediated immune responses with cellular effector functions (Gessner *et al*, 1998). The expression of each receptor class and their associated signal transduction components is tightly regulated by immune cytokines (Heijnen and van de Winkel, 1997). In patients with ITP, the production and regulation of cytokines may contribute to enhanced platelet elimination by macrophages (Andersson, 1998). For example, increased serum concentrations of interferon-gamma (IFN-γ) released during an infectious illness promote tissue macrophage Fc receptor expression and may contribute to the magnitude of the thrombocytopenia in some patients (Bussel and Schreiber, 1991). In women with ITP, hormonal modulation of macrophage Fc receptors by estrogens (upregulation) and progesterones (downregulation) is thought to contribute to exacerbations of thrombocytopenia during pregnancy (Sanders *et al*, 1987; Schreiber *et al*, 1988). The current knowledge of cytokine patterns in ITP indicate that ITP is associated with a T helper (Th)1 type of cytokine response (Andersson, 1998). This is characterized by increased serum concentrations of interleukin (IL)-2 and IFN-γ and decreased concentrations of IL-4 (Andersson, 1998). Using experimental models, this type of T cell activity has been shown to induce the activation of macrophages and cytotoxic activity in $CD8^{+}$ T cells (Andersson, 1998). In this way, dysregulation in the cytokine network in ITP may promote tissue macrophage Fc receptor expression and enhanced platelet phagocytosis (Andersson, 1998).

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studies, patients with the highest concentration of plasma IgG had the slowest reticuloendothelial clearance, whereas patients with hypogammaglobulinemia had the most rapid clearance. In patients with ITP, the low affinity Fc receptors are thought to be the most important for autoantibody-mediated platelet clearance because the high affinity Fc receptors are probably occupied with monomeric IgG (Anderson and Kelton, 1990). However, when there are high plasma concentrations of IgG, even the low affinity receptors tend to be occupied by monomeric IgG (Kelton *et al*, 1985b; Anderson and Kelton, 1990). In ITP patients, the deliberate introduction of high amounts of monomeric IgG, have proven to be therapeutically useful. In this way, treatment of ITP patients with high dose intravenous IgG can dramatically raise the concentration of IgG in the plasma and can effectively block the clearance of autoantibody-sensitized platelets (Kelton *et al*, 1985b).

1.2.3.2.2.3 Amount of IgG and complement on the cell surface

There are two mechanisms by which IgG-sensitized cells may be cleared by splenic phagocytes. Firstly, macrophages and monocytes can bind to the Fc portion of the IgG molecule and initiate phagocytosis, or secondly, IgG on the cell surface can induce clearance by activating the complement system (Anderson and Kelton, 1990). Evidence for the pathological significance of platelet surface IgG was shown by studies demonstrating that higher concentrations of platelet surface IgG correlated with platelet binding to monocytes *in vitro*(George, 1990). However, direct measurements of IgG on the platelet surface did not correlate with the degree of thrombocytopenia (Corash *et al*, 1977; Saleh *et al*, 1989). This observation is similar to that observed for autoimmune hemolytic anemia in which measurements of IgG on the surface of red blood cells did not correlate with the degree of red blood cell destruction (Constantoulakis *et al*, 1963). In both ITP and autoimmune hemolytic anemia, it is hypothesized that qualitative characteristics of both the autoantibodies and their antigenic targets,

such as their interaction with macrophages, or their importance for cell membrane integrity, are more significant determinants of the severity of disease (George, 1990; Beardsley, 1989). Alternatively, the amount of platelet-bound complement on the surface of platelets may contribute to enhanced platelet phagocytosis. Studies of IgG-sensitized red cells have indicated that when cells are coated with both IgG and complement, significantly fewer IgG molecules are required for cell destruction to occur (Anderson and Kelton, 1990). These observations suggest that some patients with ITP may have large amounts of complement products on their platelet surface, thereby requiring fewer IgG molecules for platelet phagocytosis. However, the relationship between platelet-associated IgG and platelet-associated complement in ITP is still unclear. Some investigators have reported correlations between platelet-associated IgG and complement (McMilland and Martin, 1981; Winiarski and Holm, 1983; Kurata *et al*, 1986) or between platelet-associated IgM and complement (Lehman *et al*, 1987), whereas others have found no evidence for involvement of complement (von dem Borne *et al*, 1986). These results suggest that complement may play a role in the pathophysiology of ITP in some patients, however, the precise nature of this role needs to be further clarified.

1.2.4 Pathogenetic mechanism - Potential contributing factors

1.2.4.1 Genetic predisposition

It is not clear whether there is a genetic predisposition for ITP. It has been suggested that autoimmune disorders may occur from the inheritance of subtle disorders of immune regulation, with disease expression, influenced by environmental factors (Laster *et al*, 1982; Lippman *et al*, 1982). Support for this theory has been shown by one family with documented familial ITP, in which four of six family members were affected in an autosomal dominant fashion (Karpatkin *et al*, 1981). Genetic predisposition is further supported by the occurrence of chronic ITP in monozygotic twins

(Laster *et al*, 1982). In other studies, various autoimmune disorders have been reported in relatives of patients with ITP with one study demonstrating four affected generations in one family (Lippman *et al*, 1982; Stuart *et al*, 1978). Studies of HLA typing of ITP patients are discrepant, with different groups reporting different increases in various HLA alloantigens, including; B8 and B12 (Goebel *et al*, 1977), DR-2 (Karpatkin *et al*, 1979), and Bw38 (Helmerhorst *et al*, 1982). These data clearly indicate that there is no universal association of HLA alloantigens with ITP. However, observed increases of certain HLA alloantigens in ITP patients in different geographical areas, suggests predisposing HLA alloantigens, may trigger ITP in some ethnic groups (Karpatkin, 1985).

1.2.4.2 Immunoregulation in ITP

In patients with ITP, both T-cell and B-cell abnormalities have been reported. Early studies by Trent *et al* (1981), demonstrated that T-lymphocytes from patients with chronic ITP had decreased suppressor cell activity when cultured with either autologous or homologous B-cells. More recently, it has been shown that some ITP patients have a reduction in the number of circulating $CD4^+$ suppressor-inducer cells and an increase in the number of $CD4^+$ helper-inducer cells (Semple and Freedman, 1991). These observations suggest that imperfect thymic deletion of self-reactive T-cells may play a role in the development of ITP (McMillan, 2000). Additionally, there is evidence of abnormal clonal B-cell populations in patients with ITP. In these studies, analysis of IgG glycoprotein-specific antibodies from patients with ITP demonstrated kappa or lambda light chain restriction (Christie *et al*, 1993; Stockelberg *et al*, 1995). These findings support a hypothesis of a clonal B-cell expansion producing antibodies which react with a limited number of epitopes (van der Harst *et al*, 1990; Christie e tal, 1993; Stockelberg *et al*, 1995). In other autoimmune disorders, increases in the B-cell population that expresses $CD5^+$ antigens have been reported (Abbas *et al*,

1994). In some patients with ITP, B-cells expressing CD5$^+$ antigen are markedly increased in peripheral blood and the spleen (Mizutani *et al*, 1991). However, CD5$^+$ B-cells secrete IgM autoantibodies and the majority of patients with ITP have IgG anti-platelet antibodies (Nel *et al* 1983; Cines *et al*, 1985). It has therefore been suggested that somatic hypermutation of CD5$^+$ B-cells may occur in only a small subset of ITP patients shown to have IgM autoantibodies (<20%) (Nel *et al*, 1983; Cines *et al*, 1985; Lehman *et al*, 1987) with other mechanisms of immune disruption accounting for IgG autoantibody production in the vast majority of ITP patients.

1.2.4.3 Dysregulation in the cytokine network

Investigations of serum cytokine patterns in patients with ITP suggest that ITP may occur, at least in part, as a result of a dysregulation in the cytokine network (Semple *et al*, 1996). *In vitro* studies have demonstrated that autoimmune diseases, including ITP, are associated with a Th1 type of cytokine response (Andersson, 1998). This cytokine profile is characterized by the upregulation of IL-2, IFN-γ and tumour necrosis factor (TNF)-α, and downregulation of IL-4 and IL-6 (Semple *et al*, 1996). In patients with ITP, CD4$^+$ helper T cells producing the Th1 type of cytokines, may contribute to the activation of macrophages (Semple *et al*, 1996). Alternatively, cytokines or chemokines stored within platelets themselves may play a role in the activation of macrophages. Several cytokines and chemokines, including platelet-activating factor, platelet-derived growth factor, transforming growth factor, and β-chemokines have been shown to be released from activated platelets (Power *et al*, 1995). These cytokines and chemokines serve as chemoattractors for monocytes, macrophages, granulocytes and T lymphocytes (Power *et al*, 1995). In ITP, it is speculated that platelets may become activated through cell-surface IgG interactions resulting in the release of α-granular chemokines (Andersson, 1998). The release of these chemokines may then

attract and activate macrophages which subsequently phagocytose antibody-sensitized platelets (Andersson, 1998). This hypothesis has yet to be proven.

1.3 Laboratory investigation of ITP

1.3.1 Introduction

For many years investigators have attempted to develop a sensitive and specific serological assay for the detection of platelet autoantibodies in ITP. On the basis of methodology, as well as the chronological order in which they were introduced, platelet antibody tests can be divided into three groups (Sinha and Kelton, 1990; Warner and Kelton, 1997):

Phase I assays measure a platelet-dependent end point following activation of normal platelets with ITP patient serum or plasma. In these indirect assays, the platelet-dependent end point can include platelet aggregation, platelet release, platelet lysis or platelet procoagulant formation (Warner and Kelton, 1997). Because most autoantibodies do not activate platelets, these types of assays have a low sensitivity and specificity for ITP and are currently no longer used (McMillan, 1981; Kelton and Murphy, 1990).

Phase II assays measure IgG or other proteins associated with the platelet surface or following lysis of the platelets. In these direct assays, all platelet-associated IgG is measured, including specific pathological autoantibody and IgG which is non-specifically associated with platelets. Although these assays are more sensitive than phase I assays, they lack specificity for ITP because they measure both specific and non-specific platelet-associated IgG (Warner and Kelton, 1997).

Finally, phase III assays measure IgG bound to specific platelet glycoproteins. In these direct assays, it is assumed that only the pathological antibodies are associated with specific glycoprotein targets. In this way, measurement of non-specific platelet-associated IgG is avoided. Although these assays are thought to be more diagnostically meaningful than phase I or phase II assays, they are technically difficult, time consuming, and limited by the availability of monoclonal antibodies specific for the antigenic targets of autoantibodies.

1.3.2 PAIgG Measurement

Phase II assays were first introduced in the early 1970s as a technique to measure platelet-associated immunoglobulin G (PAIgG) (Sinha and Kelton, 1990; Warner and Kelton, 1997). The ability to measure the IgG associated with platelets was thought to be a dramatic improvement over phase I assays. PAIgG assays can be subdivided into three groups, namely; direct binding assays, two-stage assays, and assays for total PAIgG.

Direct binding assays and two-stage assays measure IgG bound to the platelet surface using a monoclonal or polyclonal anti-IgG antibody or a labelled ligand. Surface PAIgG is measured either directly in a one-step incubation or calculated in a two-step procedure from the amount of residual unbound antibody or label (competitive inhibition assay). In both of these types of assays, the reported values for surface PAIgG measurements vary enormously (Sinha and Kelton, 1990). This is due in part to differences in platelet preparation, in antibody probes (or labels), and in the nature of the assays (Sinha and Kelton, 1990). In some studies reporting very high values, it has been speculated that total PAIgG was mistakenly included in the analysis of surface PAIgG. Generally,

in normal controls the amount of surface PAIgG is approximately 200 to 400 IgG molecules per platelet (Lobuglio *et al*, 1983; Court and Lobuglio, 1986).

Assays for total PAIgG are the most widely used type II assay. The popularity of these assays is primarily due to the ease of handling of the platelet specimens and the ease of fluid-phase IgG quantitation (Sinha and Kelton, 1990). In this type of assay, total PAIgG is measured following platelet lysis. In normal controls, the amount of total platelet IgG (α-granular IgG) is approximately 5 fg, or 20,000 IgG molecules per platelet (Kelton *et al*, 1979; 1985; George, 1990a). Hence, the majority of platelet IgG is found within the secretory α-granules, with less than 1-2% of the total on the platelet surface (Kelton *et al*, 1985; George *et al*, 1985; Sixma *et al*, 1984; George and Saucerman, 1988).

For many years, assays for total PAIgG were routinely used as an adjunct in the diagnosis of ITP. However, the current interpretation of elevated PAIgG values is unclear and controversial. Initially, it was assumed that autoantibodies against platelets in ITP were responsible for the increased total PAIgG. However, elevated PAIgG is also observed in thrombocytopenic disorders which are not thought to be immune-mediated. This observation challenged the assumption that all PAIgG was anti-platelet antibody. Additionally, these observations questioned the nature of PAIgG and the clinical usefulness of total PAIgG assays. Currently, phase II assays are being increasingly replaced by more diagnostically meaningful phase III assays. However, questions regarding the nature of PAIgG, and the biological interpretation of elevated PAIgG values, in both immune and non-immune thrombocytopenic disorders, have yet to be answered.

1.3.3 Elevated PAIgG in immune and non-immune thrombocytopenia

For many years, investigators using a variety of different phase II assays have consistently demonstrated that patients with ITP have increased total and surface PAIgG (Dixon *et al*, 1975; Luiken *et al*, 1977; Kelton *et al*, 1979, 1980, 1985; Hegde *et al*, 1977, 1981, 1985; Morse *et al*, 1981; Tsubakio *et al*, 1981; Kunicki *et al*, 1982; Cheung *et al*, 1983; George and Saucerman, 1988). However, elevated PAIgG is not specific for ITP and is observed in most thrombocytopenic disorders, including disorders which are not thought to be immune-mediated such as aplastic anemia and leukemia (Dixon *et al*, 1975; Luiken *et al*, 1977; Kelton *et al*, 1979, 1980, 1985; Hegde *et al*, 1977, 1981, 1985; Morse *et al*, 1981; Tsubakio *et al*, 1981; Kunicki *et al*, 1982; Cheung *et al*, 1983; George and Saucerman, 1988). In these disorders, the presence of IgG on and within platelets is typically correlated with the severity of the thrombocytopenia and not related to the mechanism responsible for the thrombocytopenia. Furthermore, measurements of total PAIgG in ITP and non-immune thrombocytopenia frequently correlate with the clinical course of the disease, decreasing when platelet counts return to normal and increasing again with recurrence of thrombocytopenia (Dixon *et al*, 1975; Luiken *et al*, 1977; Hegde *et al*, 1981).

The clinical relevance of elevated surface and total PAIgG, in immune and non-immune thrombocytopenia, are not clear. Current phase II assays for quantitating PAIgG, do not distinguish between antibody directed against a specific platelet antigen and IgG that is non-specifically adsorbed onto the platelet surface. In patients with ITP, much of the surface PAIgG is thought to be important IgG, representing pathological antibody bound to specific glycoprotein targets. In these patients, accelerated platelet clearance is mediated by pathological antibody on the platelet surface. In platelets from patients with non-immune thrombocytopenia, the origin of surface PAIgG is unclear. Since

patients with non-immune thrombocytopenia do not have a reduced platelet lifespan, it is thought that the IgG on their platelet surface is non-specific IgG and pathologically unimportant. However, the mechanism by which IgG accumulates on the surface of these platelets is unknown.

In both patients with ITP and non-immune thrombocytopenia, the majority of PAIgG is observed within the platelet. For many years, it was assumed that all α-granular proteins, including IgG, were synthesized solely by megakaryocytes. We now know that megakaryocytes endocytose plasma proteins, including IgG, and incorporate them into secretory α-granules (Handagama *et al*, 1987, 1989; Harrison *et al*, 1989). Hence some proteins, such as platelet factor 4, multimerin, and von Willebrand factor are synthesized by the megakaryocyte and routed to α-granules, whereas, other proteins, such as IgG, albumin and fibrinogen are endocytosed from the plasma, and incorporated into α-granules. In this way, it has been suggested that the majority of PAIgG within platelets represents non-specific plasma IgG with the pathologically important IgG being associated with the platelet surface.

1.3.4 Biological interpretation of increased PAIgG

Several hypotheses have been proposed to account for the observed elevations of PAIgG in patients with immune and non-immune thrombocytopenia. These hypotheses vary from proposed physical alterations of the platelet ultrastructure, to proposed functional alterations that promote platelet activation or endocytosis. Whereas some of these hypotheses account for elevated total PAIgG, others account for elevated surface PAIgG. Currently, there is no hypothesis which provides an adequate explanation for the increased amounts of PAIgG on both the platelet surface and within the platelet of patients with immune and non-immune thrombocytopenia.

1.3.4.1 Hypothesis that ITP platelets contain more α-granules

In the most currently accepted hypothesis, it is suggested that elevated PAIgG in ITP and non-immune thrombocytopenias may arise through a thrombopoietic process analogous to accelerated erythropoiesis in immune or non-immune hemolytic anemia (George, 1990). In this model, it is postulated that platelets from thrombocytopenic patients are proportionately younger and contain significantly more α-granule IgG and other α-granule proteins. Additionally, due to their increased granular content, these younger platelets may be functionally more active, thus explaining why many patients with ITP can have severe thrombocytopenia and yet no bleeding (Thompson and Jakubowski, 1988).

In this model, IgG and other plasma proteins are acquired together by fluid-phase endocytosis of whole plasma by megakaryocytes, and incorporated into α-granules. Hence, the α-granule concentration of IgG and other plasma proteins is thought to be proportional to their concentration in plasma (George *et al*, 1988). However, for any given plasma concentration, ITP platelets contain dramatically more IgG and other plasma proteins than do normal platelets. For this reason, it is proposed that the increased thrombopoietic stimulation in thrombocytopenic patients is also accompanied by a parallel increase in platelet volume (Thompson and Jakubowski, 1988; Garg *et al*, 1971; Ziegler *et al*, 1978; Holme *et al*, 1981; Levin and Bessman, 1983). In this way, patients with ITP have a greater percentage of larger, younger platelets which contain more α-granules (Penington *et al*, 1976; Martin *et al*, 1983; Corash *et al*, 1977). It would be expected that due to a greater number of α-granules per platelet, platelets from patients with ITP would contain more α-granule proteins, such as IgG and albumin (George, 1990). In this model, it would also be expected that total PAIgG and other α-granule proteins would be increased in patients with non-immune

thrombocytopenia as a result of a similar increased production of large platelets due to increased thrombopoietic stimulation (George, 1990).

In summary, this hypothesis explains observations of elevated total PAIgG in thrombocytopenic patients, however, it does not adequately explain the mechanism by which IgG accumulates on the surface of platelets from patients with non-immune thrombocytopenia.

1.3.4.2 Hypothesis that platelet activation results in elevated surface PAIgG measurements

A second hypothesis, suggests that increased amounts of IgG on the surface of platelets from thrombocytopenic patients may occur, in part, from the leakage of α-granular IgG which binds to the platelet surface (Pfueller and David, 1986). This hypothesis is based on observations which reported that the values of surface PAIgG in thrombocytopenic patients are 40 to 1000 times greater than levels of known antibodies that destroy platelets (Shulman *et al*, 1982). In this model, it is hypothesized that not all surface PAIgG is specific anti-platelet antibody and that non-specific IgG may be liberated onto the platelet surface following platelet activation, either *in vivo*, or *in vitro* during sample preparation and assay (Pfueller and David, 1986). In this way, reported observations of a linear relationship between surface and total PAIgG, in normal controls and thrombocytopenic patients, suggest that surface PAIgG values may reflect the amount of IgG within platelets rather than the amount of specific anti-platelet antibody on platelets (Pfueller and David, 1986).

Demonstrations that total PAIgG levels decrease following platelet activation with thrombin or calcium ionophore suggest that the *in vitro* release of IgG from partially activated platelets during storage or assay procedures could increase surface PAIgG levels following binding of released IgG

to the platelet surface (Pfueller and David, 1986). Previously, some studies have reported extremely high surface PAIgG values. These values were later thought to represent the mistaken inclusion of stored PAIgG in the analysis of surface PAIgG. In a similar manner, due to the very small amount of PAIgG normally associated with the platelet surface (<1% of total PAIgG), only a small amount of α-granule IgG leakage *in vitro* with subsequent binding to the platelet surface would be necessary to produce falsely elevated surface PAIgG values.

Alternatively, IgG release may occur *in vivo* following platelet activation by anti-platelet antibodies (Nomura *et al*, 1992). Studies have demonstrated that some monoclonal antibodies directed against GPIIb/IIIa appear to cause platelet activation, whereas others do not (Horsewood *et al*, 1991). In a rabbit model of immune thrombocytopenia, infused anti-platelet antibody increased the binding of non-specific IgG and albumin on the platelet surface (Sinha and Kelton, 1997). These results suggested that either platelet membrane adhesiveness was altered following Fab-dependent IgG binding or that antibody-mediated platelet activation resulted in partial α-granule release with subsequent binding of α-granular IgG and albumin to the platelet surface (Sinha and Kelton, 1997).

In summary, this hypothesis explains observations of increased surface PAIgG in thrombocytopenic patients, but fails to explain the mechanism by which total PAIgG is increased in platelets of patients with immune and non-immune thrombocytopenia.

1.3.4.3 Hypothesis of platelet endocytosis

A third hypothesis suggests that plasma proteins may accumulate in platelets by a process of receptor-mediated or fluid-phase endocytosis and that elevated PAIgG concentrations in

thrombocytopenic patients result from increased platelet endocytosis. This hypothesis is based on numerous studies demonstrating that platelets can sequester a wide variety of particulate and soluble material within channels of their surface-connected canalicular system (Movat *et al*, 1965; White, 1968; Zucker-Franklin, 1981) and the more recent evidence that platelets can incorporate exogenous proteins into their α-granules (Handagama *et al*, 1987, 1989).

1.3.4.3.1 Endocytosis of α-granule proteins

In studies by Handagama *et al* (1987, 1989), injection of horseradish peroxidase (HRP) or human plasma proteins (IgG, albumin and fibrinogen) into guinea pigs, resulted in protein uptake from the circulation and its incorporation into α-granules by bone marrow megakaryocytes. Moreover, following the injection labeled proteins were observed within the α-granules of circulating platelets more rapidly than could be explained by platelet turnover alone indicating that circulating platelets probably also endocytosed the exogenous proteins (Handagama *et al*, 1987, 1989). Similar observations were reported by Harrison *et al* (1989) who observed the time-dependent appearance of labelled fibrinogen in the megakaryocytes and platelets of an afibrinogenaemic patient given fibrinogen replacement therapy. Together these observations, provided the first evidence of an endocytic mechanism in platelets by which plasma proteins were incorporated directly into secretory α-granules.

1.3.4.3.2 Pathways of endocytosis in platelets

The mechanism by which platelets endocytose plasma proteins is poorly understood. There is evidence to suggest that both fluid-phase endocytosis and receptor-mediated endocytosis are important mechanisms of protein uptake in platelets. When platelets are incubated with fluid-phase

tracers such as thorium dioxide tracer particles can be observed to be transported in vesicles from the platelet surface to internal α- granules (Behnke, 1989). Additionally, fluid-phase markers have been observed in vesicles resembling endosomes (Behnke, 1992). These endosome-like structures appeared to originate from the surface-connected cannalicular system and subsequently fused with granules identified cytochemically as lysosomes (Behnke, 1992). Based on these observations, platelets appear to have both a degrading and a non-degrading pathway of fluid-phase endocytosis.

There is also evidence of a receptor-mediated pathway of endocytosis in platelets. In this pathway, receptor-bound macromolecules are internalized via specialized coated pits on the cell surface. This process is triggered by the binding of a ligand to a specific receptor with some receptors situated within coated pits and others moving into pits once a ligand has bound. The presence of coated pits at the surface of a cell generally indicates that receptor-mediated endocytosis occurs in the cell even if the particular receptor and its corresponding ligand are unknown (Behnke, 1989). This is true for platelets in which coated pits and coated vesicles have been morphologically identified (Behnke, 1989; Morgenstern, 1982), but the nature of the specific receptors and their corresponding ligands remains largely unknown. The one exception is the GPIIb/IIIa complex. This membrane glycoprotein, is thought to serve as the receptor for the endocytosis of fibrinogen because of several observations. Firstly, patients with Glanzmann's thrombasthenia, who are deficient in functional GPIIb/IIIa, have a corresponding decrease in platelet α-granule fibrinogen (Belloc *et al*, 1987; George *et al*, 1990). Secondly, when monkeys are injected intravenously with Fab fragments of anti-GPIIb/IIIa monoclonal antibody, platelet fibrinogen levels decrease (Suzuki *et al*, 1992). Thirdly, platelet ultrastructural studies have shown that antibody ligands to GPIIb/IIIa are internalized within

coated vesicles that later fuse with α-granules (Santoso *et al*, 1986; Morgenstern and Patscheke, 1992).

1.3.4.3.3 Endocytosis linked to platelet activation

The biological significance of the endocytotic process in platelets is still largely unknown. Several hypotheses have been proposed to explain this still poorly understood phenomena. One possibility is that platelet endocytosis is linked to platelet activation (Behnke, 1989). This hypothesis is supported by observations that fluid-phase tracers have been detected in α-granules, immediately following thrombin stimulation (Behnke, 1989), and that the number of coated vesicles found in platelets has been reported to increase following ADP stimulation (Morgenstern, 1982). Additionally, it has been reported that following activation, platelet-bound fibrinogen becomes progressively non-dissociable and is internalized to a surface-inaccessible, intracellular pool (Wencel-Drake *et al*, 1996). In this model, platelet endocytosis may serve as a regulatory mechanism that modulates platelet function (Wencel-Drake *et al*, 1996). For example, in normal platelets, the internalization of fibrinogen from the surface of activated platelets may serve to downregulate platelet adhesiveness (Wencel-Drake *et al*, 1996). In thrombocytopenic patients, the binding of IgG to the platelet surface in an Fab-dependent or nonFab-dependent manner may activate platelets resulting in receptor-mediated endocytosis and elevated total PAIgG values. Since fluid-phase endocytosis probably always accompanies receptor-mediated endocytosis, some proteins such as albumin which is found in high concentrations in plasma may be incorporated in platelet α-granules as a result of the endocytotic process.

1.3.4.3.4 Endocytosis as a defense mechanism in platelets

In patients with ITP, receptor-mediated endocytosis could be an important mechanism for clearing antibody off the platelet surface. Observations of bidirectional trafficking of membrane glycoproteins following platelet activation (Nurden *et al*, 1994; Nurden, 1997) suggest that differences in autoantibody targets in patients with ITP may determine disease severity. In this way, depending upon the antibody and its target glycoprotein, there may be differences in the fates of the antigen-associated IgG in platelets of patients with ITP. Studies have shown that GP Ib/IX is a cytoskeleton bound, non-mobile receptor and antibodies to it are not cleared from the surfaces of platelets (White *et al*, 1995, 1996, 1999). On the other hand, antibodies bound to GPIIb/IIIa are internalized by resting and activated normal platelets (Morgenstern *et al*, 1992; White *et al*, 1995) ITP platelets (Nomura *et al*, 1992) and cultured megakaryocytic cell lines (Hamamoto *et al*, 1995). Therefore, depending on the mobility of the glycoprotein target, endocytosis of autoantibody may assist the platelet in escaping sequestration and destruction by cells of the reticuloendothelial system (Santoso *et al*, 1986). Evidence for this hypothesis remains to be shown.

In summary, platelet endocytosis may serve as a mechanism by which IgG and other plasma proteins are accumulated in platelets. Endocytosis however, does not explain why IgG accumulates on the surface of platelets from patients with non-immune thrombocytopenia.

1.4 Objectives of the current investigation

For many years, elevated PAIgG was thought to be a diagnostic characteristic of ITP. However, observations that PAIgG is increased in both immune and non-immune thrombocytopenia questioned the clinical significance of PAIgG measurements and the mechanism(s) by which IgG accumulates

in platelets. The origin of IgG on the surface of platelets of patients with non-immune thrombocytopenia, is not known. Additionally, it is not clear why these IgG-sensitized platelets escape destruction by phagocytic cells of the reticuloendothelial system. In this investigation, possible biological explanations for the elevated PAIgG in adult patients with ITP and non-immune thrombocytopenia were examined. Traditionally, the laboratory investigation of ITP has focused on the development of serologic assays to measure anti-platelet autoantibodies. As an alternative investigative approach, ultrastructural techniques were used to evaluate platelets from adult patients with ITP and non-immune thrombocytopenia. Using these techniques, it was possible to localize IgG in platelets and to determine the immunomorphologic characteristics of PAIgG in patients with immune and non-immune thrombocytopenia.

The specific objectives of the current investigation were:

1. To determine whether elevated PAIgG in adult thrombocytopenic patients is a reflection of an increased platelet size with increased α-granular content.
2. To evaluate the ultrastructural morphology of platelets and the immunomorphologic characteristics of PAIgG, in adult patients with ITP and non-immune thrombocytopenia.
3. To investigate possible pathways of internalization of immune versus non-immune IgG in platelets.
4. To investigate evidence of IgG leakage onto the surface of platelets incubated *in vitro* over time.

Collectively, the results of these studies will contribute to the further understanding of a common autoimmune disorder and will provide possible biological explanations for elevated PAIgG in patients with ITP and non-immune thrombocytopenic disorders.

1.5 Thesis outline

This thesis is systematically divided into four main sections. In the first section, studies were conducted to evaluate whether elevated PAIgG in thrombocytopenic patients was a reflection of the production of larger platelets with increased α-granular content. These studies were conducted by comparing the average mean platelet volume and the measurements of different subsets of α-granular proteins in platelets from normal controls and thrombocytopenic patients. In this way, it was possible to determine whether platelets from thrombocytopenic patients were above average in both size and α-granular content.

In the second section, the ultrastructural morphology of platelets and the subcellular distribution of PAIgG in patients with ITP and non-immune thrombocytopenia were evaluated. Currently, there is limited data on the ultrastructural evaluation of platelet architecture in thrombocytopenic patients. Additionally, the immunomorphologic characteristics of PAIgG in thrombocytopenic patients, is unknown. These studies were subdivided into four sections:

1. Analysis of PAIgG following platelet disruption techniques.
1. Immunolocalization of PAIgG at the light microscopic level.
2. Quantitation of IgG in permeabilized (total IgG) and non-permeabilized platelets (surface IgG) using flow cytometry.

3. Ultrastructural evaluation of platelets and immunolocalization of PAIgG using electron microscopy.

Due to limited amounts of patient samples, it was not possible to study every patient in each study. However, platelets analyzed by electron microscopy were also analyzed using flow cytometry and confocal microscopy.

In the third section, possible pathways of IgG trafficking in platelets were investigated by determining the immunolocalization of IgG in normal platelets following incubation with anti-platelet antibody, or non-immune IgG. Using immuno-gold labeling, it was possible to quantitate the amount of IgG within various platelet organelles over time. In this way, pathways of internalization of immune versus non-immune IgG were compared.

Finally, in the fourth section, studies of IgG leakage from platelets incubated *in vitro*, were conducted. In these studies, immunomorphologic characteristics of surface PAIgG were evaluated following; (1) immediate processing or, (2) after an overnight incubation period. In this way, IgG leakage onto the surface of platelets was investigated by determining patterns of surface PAIgG distribution over time.

Acknowledgement for Chapter Two

Acknowledgement is given to Aurelio Santos for performing the antigen capture assays in the detection of anti-platelet antibodies in platelet lysate of clinic patients and normal controls.

CHAPTER 2

Measurement of endogenous and exogenous α-granular platelet proteins in patients with immune and non-immune thrombocytopenia

2.1 Introduction

A current hypothesis suggests that elevated PAIgG in patients with ITP and non-immune thrombocytopenia, is a reflection of the production of large, young platelets, in these patients (George, 1990). As a result of the increased platelet size, it is proposed that there is also an increase in α-granule content, with increased amounts of α-granule proteins, such as IgG and albumin (George, 1990). In this study, I investigated this hypothesis by comparing the average mean platelet volume and the measurements of different subsets of α-granular proteins in platelets from normal controls and thrombocytopenic patients. The strategy I employed was to compare the amount of megakaryocyte-synthesized α-granule proteins (endogenous platelet proteins; β-thromboglobulin and platelet factor 4) to the amount of plasma-derived α-granule proteins (exogenous platelet proteins; IgG, albumin and fibrinogen). I postulated that by measuring these two different subsets of α-granule proteins (endogenous and exogenous proteins) and relating these results to platelet size (mean platelet volume), I could differentiate whether the increased PAIgG in thrombocytopenic patients was due to: (1) the production of larger platelets, (2) a general overall increase in α-granular content, or (3) a selective alteration in the pool of plasma-derived α-granular proteins. In this way, it was possible to determine whether platelets from thrombocytopenic patients were above average in size and had increased α-granular content.

2.2 Materials and methods

2.2.1 Materials

All materials used for the experiments conducted within this thesis were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

2.2.2 Patients and controls

All patient studies conducted within this thesis were approved by a University and Hospital Ethics Review Committee and samples were collected with informed consent.

In this investigation, 39 adult patients with ITP were studied. Of these patients, 29 had active ITP and 10 patients were in remission. Patients were considered to have ITP if they met the following criteria; thrombocytopenia (less than 150 x 10^9/L), a nonpalpable spleen, normal or increased numbers of megakaryocytes in the bone marrow (if performed), and the absence of secondary causes of thrombocytopenia (McMillan, 1981; Kelton and Gibbons, 1982). ITP patients were subdivided into two groups, those with active disease (thrombocytopenia present) and those in remission. Patients with a past history of ITP and normal platelet counts (>150 x 10^9/L) who were not on any immunosuppressive therapy at time of investigation were classified as having ITP in remission. All ITP patients were tested in an antigen capture assay to determine the presence of antibodies bound to GPIIb/IIIa or GPIb/IX.

The control patients (n=60) with other hematological disorders included adult patients with multiple myeloma, aplastic anemia, thrombotic thrombocytopenic purpura, systemic lupus erythematosus, iron deficiency anemia, myeloproliferative disorder, hypersplenism, splenomegaly, leukemia, lymphoma,

alcoholic liver cirrhosis and incidental thrombocytopenia of pregnancy. These patients were subdivided according to their platelet counts (thrombocytopenic, n=26; normal platelet count, n=34). The normal controls (n=13) were a group of nonthrombocytopenic, aspirin-free, healthy male and female laboratory personnel. All control patients and normal controls were tested in an antigen capture assay to determine the presence of antibodies bound to GPIIb/IIIa or GPIb/IX.

2.2.3 Platelet collection and preparation for antigen capture assay

The detection of anti-GPIIb/IIIa and anti-GPIb/IX antibodies was conducted using the antigen capture (AC) assay according to Warner *et al* (1999). Briefly, whole blood was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1 mM theophylline and prostaglandin E_1 (1 ug/ml). Platelet-rich plasma (PRP) was obtained by centrifugation at 160 x g for 20 minutes at room temperature. Positive control platelets were prepared by incubating PRP with serum from a patient with post-transfusion purpura (PTP), containing anti-Pl^{A1} antibodies (anti-GPIIb/IIIa). Platelets were isolated by differential centrifugation and washed three times in PBS-ACD (with 1 mM theophylline, 1.5 mM prostaglandin E_1, pH 6.2). Platelets were resuspended to a final concentration of 300,000/ul in lysing buffer (25 mM Tris, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid disodium (EDTA), 1% bovine serum albumin (BSA), 5 mg/ml soybean trypsin inhibitor, 1.5 mM sodium azide, 0.25 mM phenylmethylsulfonyl fluoride (PMSF)), and lysed by the addition of Triton X-100 (1%) and SDS (0.1%).

2.2.4 Antigen capture assay

Immulon microtiter wells (Dynatech, Chantilly, VA) were coated overnight with 100 ul of a 10 ug/ml solution of Raj-1 (anti-GPIIb/IIIa) or BEB-1 (anti-GPIb/IX), in carbonate buffer (35 mM sodium

hydrogen carbonate, 15 mM sodium carbonate, pH 9.6) (Horsewood *et al*, 1991). Wells were blocked with 250 ul blocking buffer (50 mM Tris, 0.25 mM NaCl, 0.05% Tween, 2% BSA, pH 7.4) for two hours at room temperature, and washed with wash buffer (50 mM Tris, 0.25 mM NaCl, 0.05% Tween, pH 7.4). Duplicate 50 ul samples of platelet lysate were added to the wells and incubated for 3 hours at room temperature. Wells were washed 3 times with wash buffer and 100 ul alkaline phosphatase-conjugated affinity-purified F(ab')$_2$ fragment goat anti-human IgG (Fcγ) antibody (1/1000) (Jackson Immuno Research Laboratories Inc, Philadelphia, PA) were added per well. Following a one-hour incubation at room temperature, wells were washed three times with wash buffer and secondary antibody was detected by the addition of 100 ul/well of p-nitrophenylphosphate (1 mg/ml) in 1 M diethanolamine buffer. Reactions were terminated at 20 minutes using 0.5 N H_2SO_4 and absorbance was measured at a wavelength of 405 nm.

2.2.5 Platelet collection and preparation for quantitative protein analysis

Whole blood was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1 mM theophylline and prostaglandin E_1 (1 ug/ml). Platelet-rich plasma (PRP) was obtained by centrifugation at 160 x g for 20 minutes at room temperature. The platelet pellet was then isolated by centrifugation at 2000 x g for 10 minutes and washed 5 times in phosphate buffered saline (PBS, pH 6.5) containing 10 mM ethylenediaminetetra-acetic acid disodium (EDTA). Washed platelets were resuspended to 1 x 10^8 platelets/ml in buffer (25 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.02% BSA), containing the protease inhibitors, leupeptin (50 ug/ml) and PMSF (0.25 mM). Platelets were lysed by the addition of Triton X-100 (0.5% final) (Pierce, Rockford, IL). The lysed platelets were centrifuged at 15 000xg for 10 minutes to remove insoluble proteins before quantitative analysis.

2.2.6 Measurement of platelet-associated IgG (PAIgG)

PAIgG was measured in platelet lysates using an immunoradiometric assay. Anti-human IgG monoclonal antibody CAG-2, raised in our laboratory, was coated onto Immulon II (Dynatech, Chantilly, VA) microtitre strip wells in 0.2 mol/L carbonate buffer (pH 9.5) overnight at 4°C. This antibody recognizes all subclasses of IgG. The wells were washed once with wash buffer (0.9% NaCl, 0.05% Tween-20) and blocked with 2% BSA/PBS for 2 hours at room temperature. Platelet lysates were diluted (1.25 - 6.25 x 10^6 platelets/ml) in 2% BSA/PBS and duplicate 100 ul samples were added to the wells. Lysates were incubated for 3 hours at room temperature and wells washed five times with wash buffer.

The bound IgG was detected using monoclonal anti-human IgG, HB43 (2 mg/ml) (American Type Culture Collection, Rockville, MD). This antibody was radio-labeled with 1 mCi $Na^{125}I$ using the chloramine-T method and had an average specific activity of 189 mCi/mg protein. The ^{125}I-HB43 was diluted (1/1000) in 2% BSA/PBS and 100 ul was added to each well. Following an incubation of 2 hours at room temperature, the wells were washed 5 times with wash buffer and each well was transferred into 12 x 75 mm plastic tubes (Sarstedt, Newton, NC). An LKB 1275 minigamma counter (Fisher Scientific, Nepean, ON) was used to determine counts per minute per tube. Purified human IgG (Sigma-Aldrich, Oakville, ON) was diluted in 2% BSA/PBS with Triton X-100 (0.5% final), and used to generate standard curves. Results were expressed as ug IgG/10^9 platelets, equivalent to fg IgG/platelet.

2.2.7 Measurement of platelet-associated albumin

Platelet-associated albumin was measured using a similar method. Wells were coated with monoclonal anti-human albumin (1.8 mg/ml) (Clone HSA1/25.1.3; Cedarlane Laboratories, Hornby, ON) in carbonate buffer (pH 9.6) overnight at 4°C. The wells were washed once with wash buffer and blocked with 2% BSA/PBS for 2 hours at room temperature Platelet lysates were diluted (1.5 - 6.25 x 10^6 platelets/ml) in 2% BSA/PBS and duplicate 100 ul samples were added to plate wells. Lysates were incubated for 3 hours at room temperature and wells washed five times with wash buffer.

The bound albumin was detected using a second monoclonal anti-human albumin antibody (2 mg/ml) (Clone 943127; Cortex Laboratories, San Leandro, CA). This antibody was radio-labeled with 1 mCi $Na^{125}I$ (average specific activity 505 mCi/mg protein), diluted (1/1000) in 2% BSA/PBS, and 100 ul was added to each well. Following an incubation of 2 hours at room temperature, wells were washed 5 times with wash buffer. Each well was placed into 12 x 75 mm plastic tubes and counted in a gamma counter. The two monoclonal anti-human albumin antibodies used in this assay recognized human but not bovine, serum albumin. Purified human albumin (Sigma-Aldrich, Oakville, ON) diluted in 2% BSA/PBS, with Triton X-100 (0.5% final), was used to generate standard curves. Results were expressed as ug albumin/10^9 platelets.

2.2.8 Measurement of β-thromboglobulin and platelet factor 4

Beta-thromboglobulin (β-TG) and platelet factor 4 (PF4) were measured in platelet lysates using commercial enzyme-linked immunosorbent assay kits obtained from Asserachrom (Murex

Diagnostics, Guelph, ON), according to the manufacturer's specifications. β-TG and PF4 concentrations were expressed as ug β-TG or PF4/10^9 platelets.

2.2.9 Measurement of platelet-associated fibrinogen

Platelet-associated fibrinogen was measured using an enzyme-linked immunosorbent assay, described previously (Hayward *et al*, 1996). Briefly, wells were coated with goat anti-human fibrinogen (1 ug IgG/well) (Organon Teknika, Scarborough, ON) in carbonate buffer (pH 9.6) overnight at 4°C. The wells were washed once with wash buffer and blocked with 2% BSA/PBS for 2 hours at room temperature. Platelet lysates were diluted in 2% BSA/PBS and duplicate 100 ul samples were added to plate wells. Lysates were incubated for 3 hours at room temperature, and wells washed five times with wash buffer. The bound fibrinogen was detected using rabbit anti-human fibrinogen (2.4 mg/ml) (1/1000) (Behring Diagnostics Inc., Westwood, MA) followed by alkaline-phosphatase-conjugated goat anti-rabbit IgG (1/1000) (BioCan Scientific Inc., Mississauga, ON) and p-nitrophenyl phosphate substrate tablets (OD_{405}). Dilutions of purified human fibrinogen (Alexis Biochemicals, San Diego, CA), in 2% BSA/PBS, with Triton X-100 (0.5% final) were used to generate standard curves. The results were expressed as ug fibrinogen/10^9 platelets.

2.2.10 Mean platelet volume

Blood samples were collected into EDTA anticoagulant tubes (Becton Dickinson, Mississauga, ON) and analyzed on a Coulter S-Plus Counter (Beckman Coulter, Hialeah, FL), for determination of mean platelet volume (fl).

2.2.11 Serum IgG and serum albumin measurements

Serum IgG and albumin were measured using a Kallestad QM 300 Protein Analysis System (Sanofi Diagnostics Pasteur, Chaska, MN).

2.3.12 Plasma fibrinogen measurements

Plasma fibrinogen levels were measured using the Clauss fibrinogen assay and an MLA Electra 1600C Automatic Coagulation Analyzer (Medical Laboratory Automation, Montreal, QC).

2.3.13 Statistical analysis

Statistical analyses were performed using the Corel Quattro Pro (Version 7) data analysis program. Inferences about the corresponding measurements of endogenous and exogenous alpha granular platelet proteins, total platelet protein and mean platelet volume, for each patient group, was made by calculating the mean and standard deviation for each data set. The quantitative upper limit of normal for each platelet protein measured, was based on the data set of the healthy control group (mean + 2 standard deviations). The Pearson product moment coefficient of correlation, r, was calculated to determine whether data sets were correlated. In addition, the calculated mean for each data set was compared by using an analysis of variance (ANOVA, one-way) to determine whether differences among the population means were significant ($p<0.05$).

2.3 Results

Using an antigen capture assay, platelet lysates from patients with ITP (29/29) tested positive for anti-GPIIb/IIIa or anti-GPIb/IX antibodies. Platelet lysates from patients with ITP in remission (10/10), or non-immune hematological disorders (60/60) (thrombocytopenic, n=26; normal platelet

counts, n=34) were negative for anti-GPIIb/IIIa or anti-GPIb/IX antibodies. Similarly, all normal control platelet lysates (13/13) were negative for anti-GPIIb/IIIa or anti-GPIb/IX antibodies.

The defined quantitative upper limit of normal for each platelet protein measured was based on values obtained from a group of healthy controls (n=13). The results obtained were similar to the results obtained by other investigators using different techniques. Together, these studies indicate that the normal amounts of protein from solubilized platelets are less than 5 ug PAIgG/10^9 platelets (Kelton and Steeves, 1983; Kelton *et al*, 1989), less than 15 ug albumin/10^9 platelets (Kelton and Steeves, 1983), 80-140 ug fibrinogen/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993), 30 to 80 ug β-TG/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993; Bellon *et al*, 1993; Kerry and Curtis, 1985) and 1 to 14 ug PF4/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993; Bellon *et al*, 1993; Kerry and Curtis, 1985).

In patients with immune and non-immune thrombocytopenia, mean values of plasma-derived α-granular proteins (IgG, albumin and fibrinogen) were significantly higher than those observed in patients with normal platelet counts (including ITP in remission and non-immune hematological disorders) ($p<0.05$) (Figures 1-3). The mean values and standard deviations for these measurements are summarized in Table 1. These elevations were consistent and independent of the mechanism responsible for the thrombocytopenia. The one exception was observed in the group of patients with non-immune hematological disorders and normal platelet counts. In this group of patients, mean platelet fibrinogen was significantly elevated, compared to normal controls ($p=0.0004$). In all patient groups, patients with elevated PAIgG, platelet-associated albumin and/or platelet fibrinogen had normal levels of serum IgG, serum albumin and plasma fibrinogen.

When the megakaryocyte synthesized platelet proteins (β-TG and PF4) were measured in the group of immune and non-immune thrombocytopenic patients, the mean values for these proteins were not significantly elevated, compared to normal controls ($p>0.05$) (Figure 4). Despite, measured elevations in the amount of plasma-derived α-granular proteins, a parallel increase in megakaryocyte-synthesized α-granular proteins, was not observed (Table 1). These observations were similar for patients with normal platelet counts in which levels of megakaryocyte-synthesized proteins were not elevated (Figure 4).

When the average mean platelet volume was determined for each patient group, values were within the established normal range (Figure 5). Additionally, there was no correlation between mean platelet volume and amount of PAIgG, in platelets from ITP patients ($r = -0.23$) or platelets from patients with non-immune thrombocytopenia ($r = -0.26$). Similarly, there was no correlation between mean platelet volume and amount of platelet albumin in platelets from both thrombocytopenic patient groups ($r<0.15$).

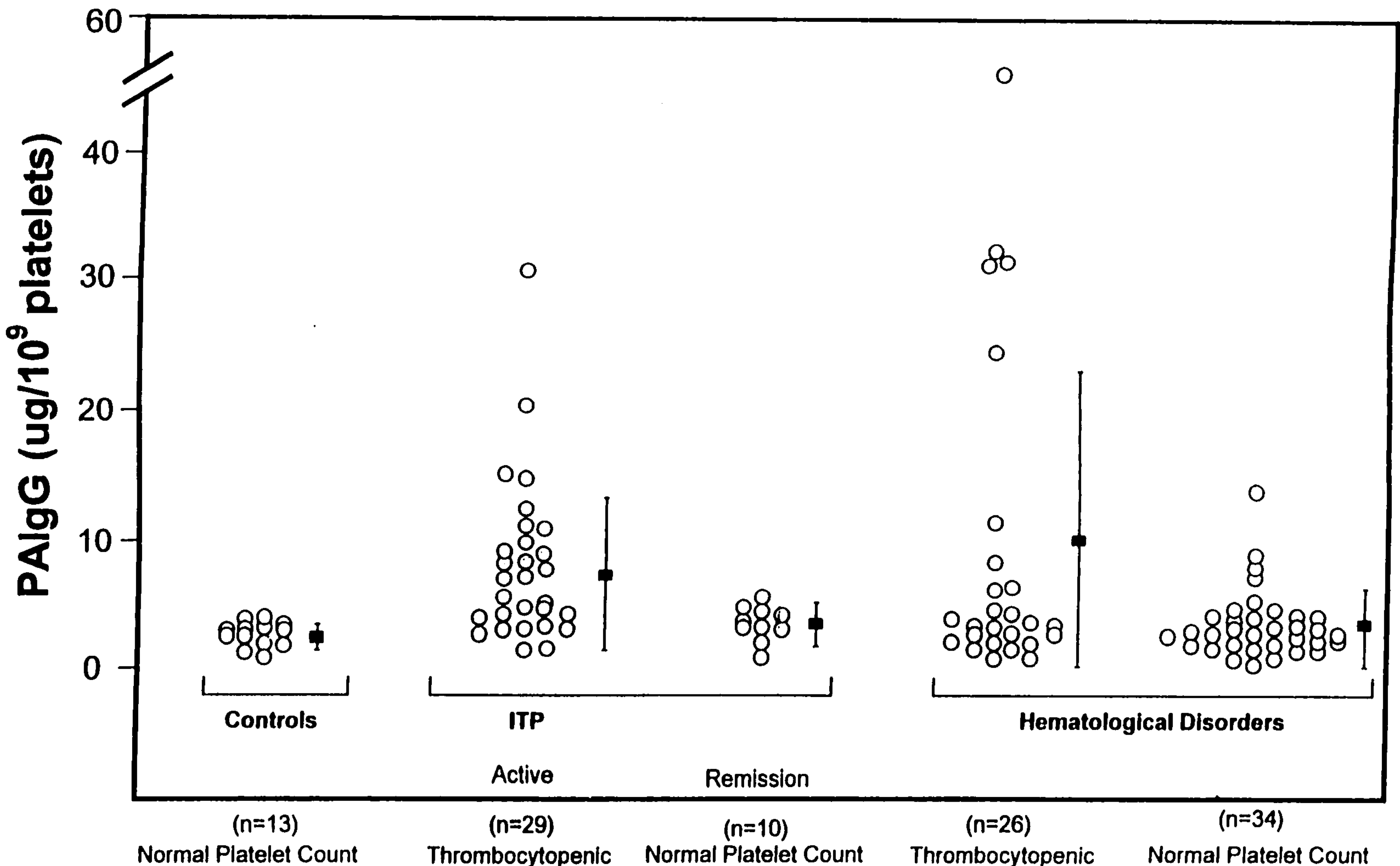
TABLE 1. **Measurement of endogenous and exogenous alpha granular platelet proteins, and platelet size in thrombocytopenic patients and patients with normal platelet counts.**

	Platelet size	Plasma-derived platelet proteins			Megakaryocyte synthesized proteins	
	MPV (fl)	**IgG** (ug/10^9 platelets)	**ALBUMIN** (ug/10^9 platelets)	**FIBRINOGEN** (ug/10^9 platelets)	**BTG** (ug/10^9 platelets)	**PF4** (ug/10^9 platelets)
ACTIVE ITP (Thrombocytopenic) (n=29)	10.1 ± 1.3	***7 ± 6.1***@	***21.9 ± 18.3***#	***547 ± 644***$	67.7 ± 32.4	3.1 ± 1.3
ITP - REMISSION (Normal Platelet Count) (n=10)	8.9 ± 0.7	3.1 ± 1.2	10.5 ± 3.4	155 ± 143	55.6 ± 26.2	2.6 ± 1.1
NONIMMUNE HEMATOLOGICAL DISORDERS (Thrombocytopenic) (n=26)	8.3 ± 1.5	***10.3 ± 13.8****	***36.2 ± 46.8***&	***420 ± 494***~	51.4 ± 28.6	2.5 ± 1.3
NONIMMUNE HEMATOLOGICAL DISORDERS (Normal Platelet Count) (n=34)	8.6 ± 1.8	3.4 ± 2.4	9.1 ± 9.3	***336 ± 387***^	68.9 ± 63.1	3.4 ± 2.2
HEALTHY CONTROLS (Normal Platelet Count) (n=13)	9.7 ± 0.7	2.4 ± 0.9	6.4 ± 1.6	130 ± 52	35.6 ± 23.2	2.3 ± 1.2
DEFINED UPPER LIMITS OF NORMAL (MEAN + 2 SD) (Based on healthy controls)	*11.1*	*4.3*	*9.6*	*234.8*	*82*	*4.7*

Data are reported as means +/- standard deviations, n=number of samples. Italicized values are those which were significantly higher than the defined upper limit of normal for that protein; @p=0.00007, # p=0.0017, $ p=0.0029, * p=0.0026, & p=0.0038, ~ p=0.0026, ^ p=0.0004.

Figure 1 Measurement of PAIgG in ITP and non-immune thrombocytopenia.

PAIgG in platelet lysate (ug IgG/10^9 platelets) was measured using an immunoradiometric assay. Each symbol represents a single patient or healthy control. The mean (black square) and standard deviation for each patient or healthy control group is indicated.



PAIgG (ug/10^9 platelets)
60
40
30
20
10
0
Controls
ITP
Hematological Disorders
Active
Remission
(n=13)
Normal Platelet Count
(n=29)
Thrombocytopenic
(n=10)
Normal Platelet Count
(n=26)
Thrombocytopenic
(n=34)
Normal Platelet Count

Figure 2 Measurement of platelet *al*bumin in ITP and non-immune thrombocytopenia.

Platelet-associated albumin in platelet lysate (ug albumin/10^9 platelets) was measured using an immunoradiometric assay. Each symbol represents a single patient or healthy control. The mean (black square) and standard deviation for each patient or healthy control group is indicated.

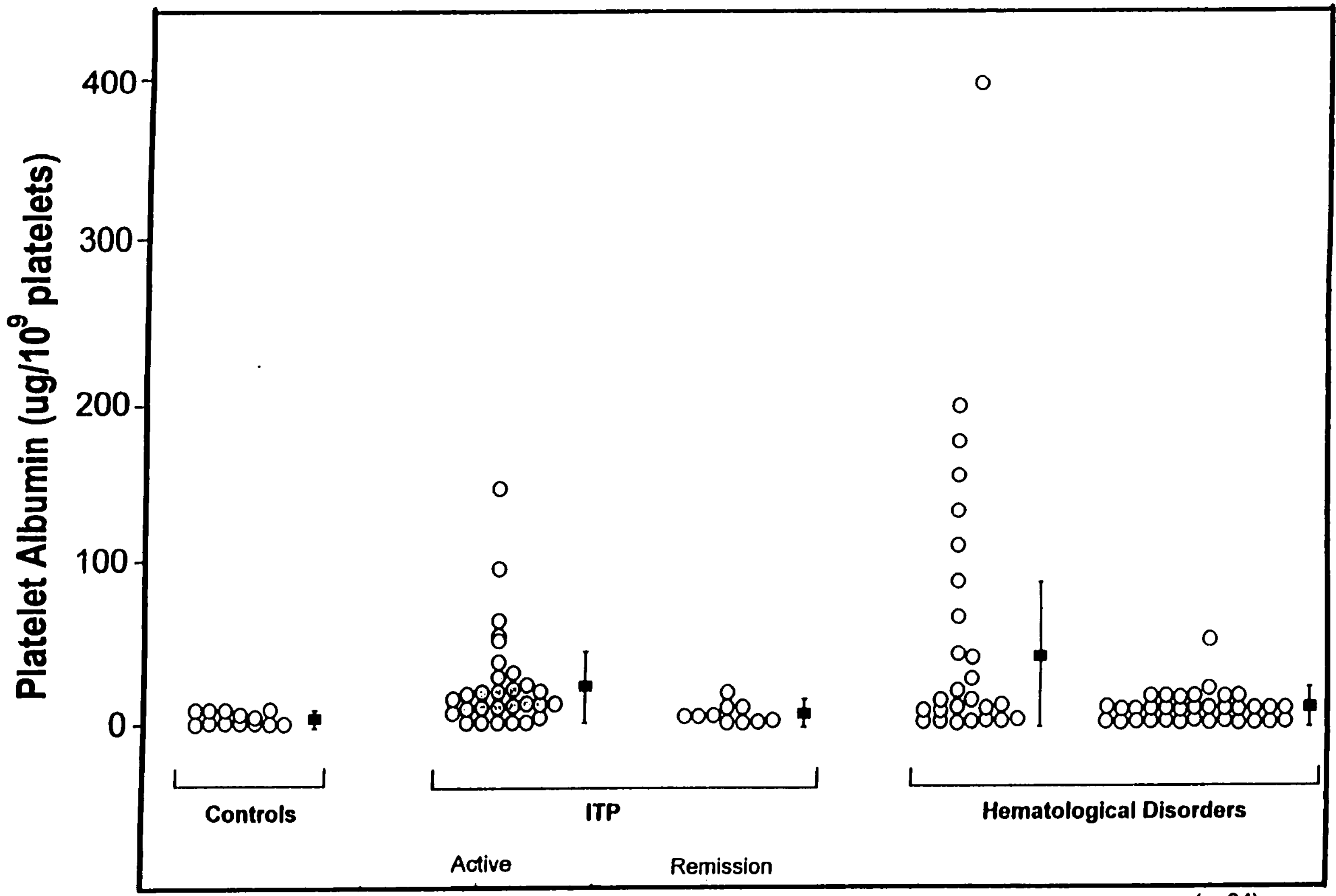
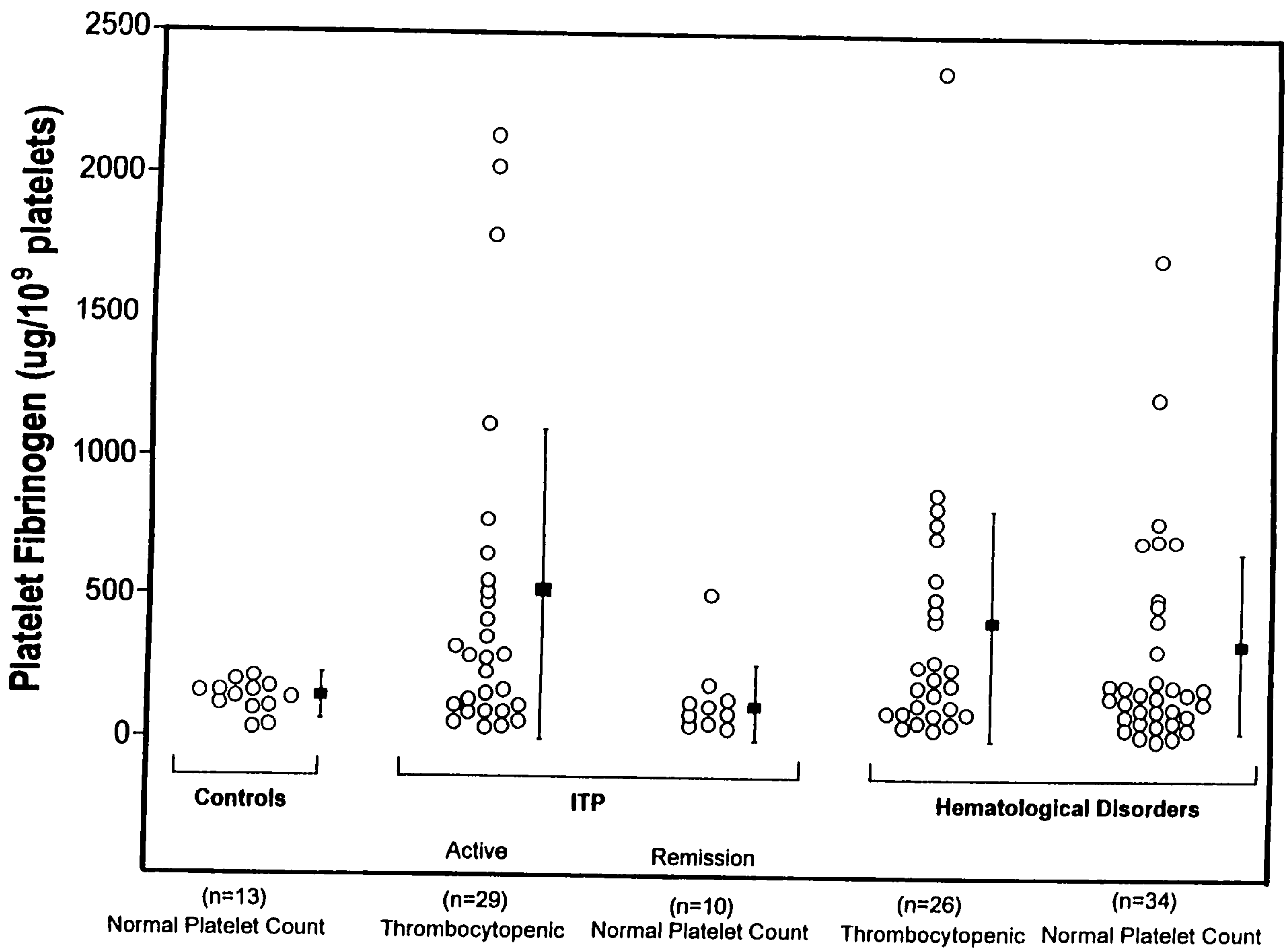

Platelet Albumin (ug/10^9 platelets)
400
300
200
100
0
Controls
ITP
Hematological Disorders
Active
Remission
(n=13)
Normal Platelet Count
(n=29)
Thrombocytopenic
(n=10)
Normal Platelet Count
(n=26)
Thrombocytopenic
(n=34)
Normal Platelet Count

Figure 3 Measurement of platelet fibrinogen in ITP and non-immune thrombocytopenia.

Platelet-associated fibrinogen in platelet lysate (ug fibrinogen/10^9 platelets) was measured using an enzyme-linked immunosorbent assay. Each symbol represents a single patient or healthy control. The mean (black square) and standard deviation for each patient or healthy control group is indicated.



Platelet Fibrinogen (ug/10^9 platelets)
2500
2000
1500
1000
500
0
Controls
ITP
Hematological Disorders
Active
Remission
(n=13)
Normal Platelet Count
(n=29)
Thrombocytopenic
(n=10)
Normal Platelet Count
(n=26)
Thrombocytopenic
(n=34)
Normal Platelet Count

Figure 4 Measurement of platelet factor 4 (PF4) and beta-thromboglobulin (βTG) in ITP and non-immune thrombocytopenia.

Platelet factor 4 (PF4) and beta-thromboglobulin (βTG) in platelet lysate (ug/10^9 platelets) were measured using enzyme-linked immunosorbent assays. Each symbol represents a single patient or healthy control. The mean (black square) and standard deviation for each patient or healthy control group is indicated.

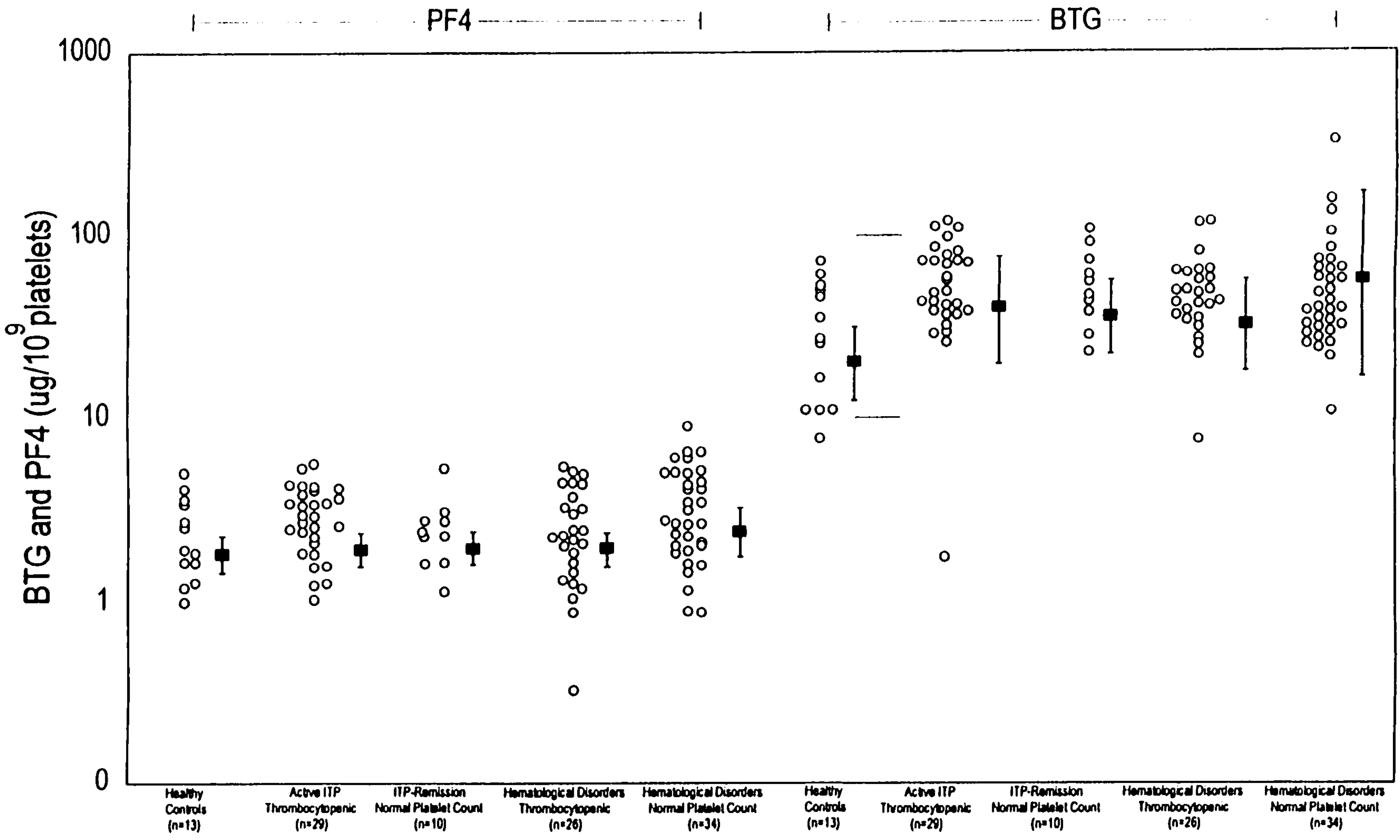
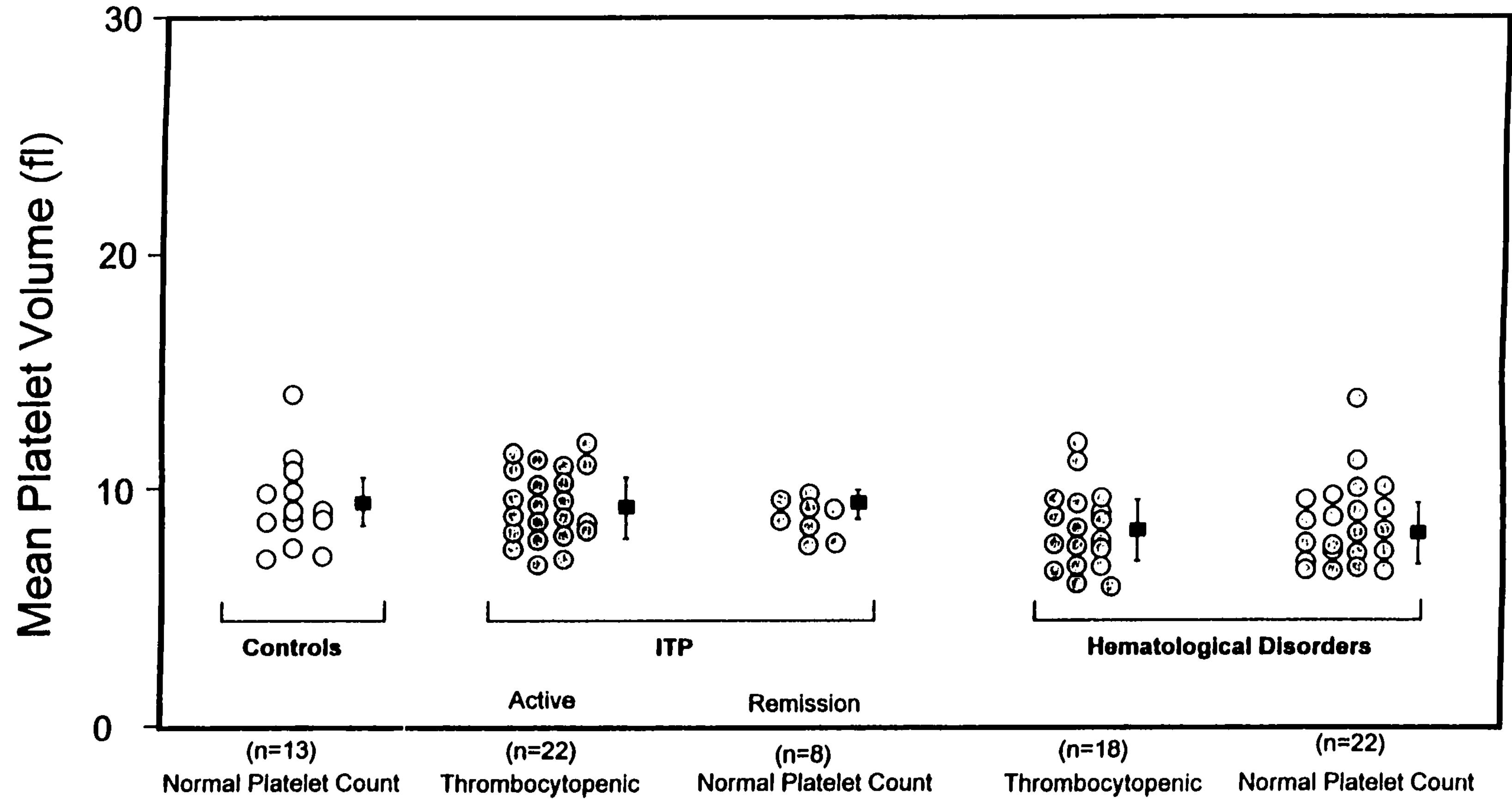

PF4
BTG
BTG and PF4 (ug/10^{9}platelets)
1000
100
10
1
0
Healthy Controls (n=13)
Active ITP Thrombocytopenic (n=29)
ITP-Remission Normal Platelet Count (n=10)
Hematological Disorders Thrombocytopenic (n=26)
Hematological Disorders Normal Platelet Count (n=34)
Healthy Controls (n=13)
Active ITP Thrombocytopenic (n=29)
ITP-Remission Normal Platelet Count (n=10)
Hematological Disorders Thrombocytopenic (n=26)
Hematological Disorders Normal Platelet Count (n=34)

Figure 5 Measurement of mean platelet volume (MPV) in ITP and non-immune thrombocytopenia.

Mean platelet volume (fl) was determined by electronic sizing on a Coulter S-Plus Counter. Each symbol represents a single patient or healthy control. The mean (black square) and standard deviation for each patient or healthy control group is indicated.


Mean Platelet Volume (fl)
30
20
10
0
Controls
ITP
Active
Remission
Hematological Disorders
(n=13)
Normal Platelet Count
(n=22)
Thrombocytopenic
(n=8)
Normal Platelet Count
(n=18)
Thrombocytopenic
(n=22)
Normal Platelet Count

2.4 Summary

In this study, I investigated the hypothesis that elevated PAIgG in patients with ITP and non-immune thrombocytopenia, is a reflection of the production of large, young platelets, with increased α-granule protein content, including platelet IgG (George and Saucerman, 1988; George, 1989). By comparing the average mean platelet volume, and measuring two subsets of alpha granule proteins, it was possible to determine whether platelets from thrombocytopenic patients were notably above average in size with increased α-granular proteins. Based on their origin of synthesis, the proteins measured included megakaryocyte-synthesized (endogenous α-granular proteins) and plasma-derived (exogenous α-granular proteins) platelet proteins.

The defined quantitative upper limit of normal for each platelet protein measured was based on values obtained from a group of healthy controls (n=13). The results I obtained were similar to the results obtained by other investigators using different techniques. Together these studies indicate that the normal amounts of protein from solubilized platelets are less than 5 ug PAIgG/10^9 platelets (Kelton and Steeves, 1983; Kelton *et al*, 1989), less than 15 ug albumin/10^9 platelets (Kelton and Steeves, 1983), 80-140 ug fibrinogen/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993), 30 to 80 ug β-TG/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993; Bellon *et al*, 1993; Kerry and Curtis, 1985) and less than 5 to 14 ug PF4/10^9 platelets (Colman *et al*, 1994; McKeown *et al*, 1993; Bellon *et al*, 1993; Kerry and Curtis, 1985).

The measurement of platelet proteins in platelet lysates, demonstrated elevated amounts of plasma-derived α-granular proteins in lysates from patients with thrombocytopenia with no parallel increase in megakaryocyte synthesized proteins. These elevations were not associated with elevated plasma

levels of these proteins or increased mean platelet volume. Additionally, these elevations were consistent and irrespective of whether the thrombocytopenia was caused by immune or non-immune mechanisms. These results demonstrate that elevated platelet IgG, albumin and fibrinogen in thrombocytopenic patients represents a selective increase in the pool of plasma-derived α-granular proteins and not an overall increase in α-granular content or platelet size.

The results of this study have been published in the British Journal of Haematology.

MEASUREMENT OF ENDOGENOUS AND EXOGENOUS ALPHA-GRANULAR PLATELET PROTEINS IN PATIENTS WITH IMMUNE AND NON-IMMUNE THROMBOCYTOPENIA.

Mary Hughes, Catherine P.M. Hayward, Peter Horsewood, Theodore E. Warkentin and John G. Kelton. BRITISH JOURNAL OF HAEMATOLOGY (1999) 106:762-770.

CHAPTER 3

Investigations of the ultrastructural morphology of platelets from patients with ITP and non-immune thrombocytopenia, and the subcellular localization of PAIgG

Part 1 Analysis of PAIgG following platelet disruption techniques

3.1 Introduction

For many years, PAIgG has been measured on the platelet surface of patients with immune and non-immune thrombocytopenia using phase II assays. Currently, the origin of elevated surface PAIgG in patients with non-immune thrombocytopenia is unknown. Additionally, the observed variability in surface PAIgG values has led to confusion and skepticism concerning the methodology of these assays. It remains uncertain how best to measure surface PAIgG. In this first of a series of investigations, platelet disruption techniques were utilized to measure PAIgG in platelet membrane fractions of patients with immune and non-immune thrombocytopenia. In this way, potential problems of measuring IgG on intact platelets was avoided. The purpose of these studies was to investigate the compartmentalization of PAIgG in platelets from patients with immune and non-immune thrombocytopenia. By measuring the amount of membrane-associated PAIgG compared to the amount of soluble PAIgG (presumably, α-granular PAIgG), the gross subcellular distribution of PAIgG in thrombocytopenic patients could be determined. In these studies, the platelet membrane fraction referred to the insoluble platelet fraction or the centrifugable platelet fraction versus the soluble fraction or non-centrifugable fraction.

3.1.1 Materials and methods

3.1.1.1 Patients and controls

In this investigation, 9 adult patients with ITP, 14 adult patients with non-immune hematological disorders (thrombocytopenic, n=7; normal platelet count, n=7), and 7 healthy adult controls were studied. Patients categorized with non-immune thrombocytopenia were all recovering from bone marrow transplants and had not received platelet or whole blood transfusions at the time of study. In an antigen capture assay (outlined in section 2.2.4), platelet lysates from all patients with ITP demonstrated detectable anti-GPIIb/IIIa or anti-GPIb/IX antibodies, whereas all non-immune patients and normal controls tested negative.

3.1.1.2 Platelet collection and preparation

Whole blood was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1 mM theophylline and prostaglandin E_1 (1 ug/ml). Platelet-rich plasma (PRP) was obtained by centrifugation at 160 x g for 20 minutes at room temperature. Platelets were isolated by differential centrifugation and washed 3 times in phosphate buffered saline (PBS, pH 6.5) with 10 mM EDTA. Washed platelets were resuspended to 1 x 10^8 platelets/ml in buffer (25 mM Tris, 100 mM NaCl, 10 mM EDTA), containing the protease inhibitors, leupeptin (50 ug/ml) and phenylmethylsulfonyl fluoride (PMSF) (0.25 mM). For each platelet sample, a subsample was taken to measure PAIgG in total platelet lysate as described in section 2.2.6. In preliminary experiments examining the effectiveness of several platelet disruption techniques, platelets were incubated for 30 minutes at 37°C with radiolabels (^{14}C-serotonin and ^{111}Indium oxine) before platelet disruption.

3.1.1.3 Platelet disruption technique

Preliminary experiments using platelets labeled with ^{111}Indium oxine (Chedoke-McMaster Hospitals, Nuclear Pharmacy, Hamilton, ON), ^{14}C-serotonin (Amersham, Oakville, ON) or measurement of platelet factor 4 (as described in section 2.2.8) were conducted to compare several methods of platelet disruption. Disruption methods included nitrogen bomb cavitation, sonication and repeated cycles of freeze-thawing. Disruption of platelets by nitrogen bomb cavitation was performed by pipetting the platelet suspension into the chamber of a Parr cell disruption bomb (Parr Instrument Co. Moline, IL) attached to a cylinder of nitrogen gas. Using a high pressure regulator, platelets were disrupted by applying a constant gas pressure of 1200 psi for 5 minutes according to Broekman (1992). Disruption of platelets by sonication was conducted using a micro-ultrasonic cell disrupter (Kontes, Richmond, CA). Briefly, 3 ml of platelet suspension were pipetted into a plastic 15 ml tube and chilled in a beaker of ice-water for 10 minutes. The sonicator probe was placed into the platelet suspension and short 3 second bursts (15 in total) were applied over a 3 minute period with constant chilling according to Neubig and Szamraj (1986). Finally, disruption of platelets by repeated freeze/thawing was performed by pipetting 3 ml of platelet suspension into a 15 ml plastic tube and rapidly freezing the platelet suspension in a bath of dry-ice and ethanol (-70°C) followed by thawing at 37°C. This freeze/thaw cycle was repeated 2 to 20 times. A sample of disrupted platelets was fixed in 1% glutaraldehyde and embedded in LR White resin for analysis under an electron microscope.

3.1.1.4 Isolation of the platelet membrane fraction from the soluble fraction

Following platelet disruption, platelet suspensions were pipetted into ultracentrifuge tubes (Beckman, Palo Alto, CA) and ultracentrifuged at 100, 000 x g for 1 hour at 4°C in an L8-80 Beckman Ultracentrifuge. The platelet supernatant (soluble fraction) was removed and the platelet membrane

pellet was washed in platelet wash buffer [25 mM Tris, 100 mM NaCl, 10 mM EDTA containing the protease inhibitors leupeptin (50 ug/ml) and PMSF (0.25 mM)]. The washed platelet membrane was ultracentrifuged and solubilized by the addition of Triton X-100 (0.5% final) (Pierce, Rockford, IL). Identical concentrations of Triton X-100 were added to supernatant fractions.

3.1.1.5 Measurement of PAIgG

PAIgG was measured in platelet lysates, solubilized platelet membrane fractions and platelet supernatant fractions (soluble platelet fraction) using an immunoradiometric assay as described in section 2.2.6.

3.1.1.6 Statistical analysis

Statistical analyses were performed using the Corel Quattro Pro (Version 7) data analysis program. Inferences about the corresponding measurements of PAIgG in platelet membrane and supernatant fractions for each patient group were made by calculating the mean and standard deviation for each data set. The quantitative upper limit of normal for each platelet fraction was based on the data set of the healthy control group (mean + 2 standard deviations). The calculated means for each data set were compared by using an analysis of variance (ANOVA, one-way) to determine whether differences among the population means were significant ($p<0.05$).

3.1.2 Results

Platelet membranes are extensive and include, the platelet surface membrane, the membrane channels of the surface-connected cannalicular system, and the intracellular membranes of platelet organelles. In normal platelets, the majority of PAIgG is found within α-granules. For this reason,

preliminary experiments were conducted to determine the degree of platelet disruption necessary for sufficient disruption of both external platelet surface membrane and intracellular α-granule membrane. As markers of intact external and intracellular membranes, platelets were labeled with ^{111}-Indium oxine, a marker of the platelet cytoplasm and ^{14}C-serotonin, a marker of platelet dense granules. A third marker, platelet factor 4 (PF4), an endogenous α-granule protein, was measured as a marker of intact α-granules.

A preliminary comparison of different cell disruption techniques, using using ^{14}C-serotonin labeled platelets demonstrated that less than 3% of label was associated with the platelet membrane fraction following repeated freeze-thawing. Platelet disruption using sonication and nitrogen cavitation were less effective, with 20% and 50% of label associating with the membrane fraction, respectively. Based on these results, platelets were routinely disrupted by repeated freeze-thawing (10 cycles in total).

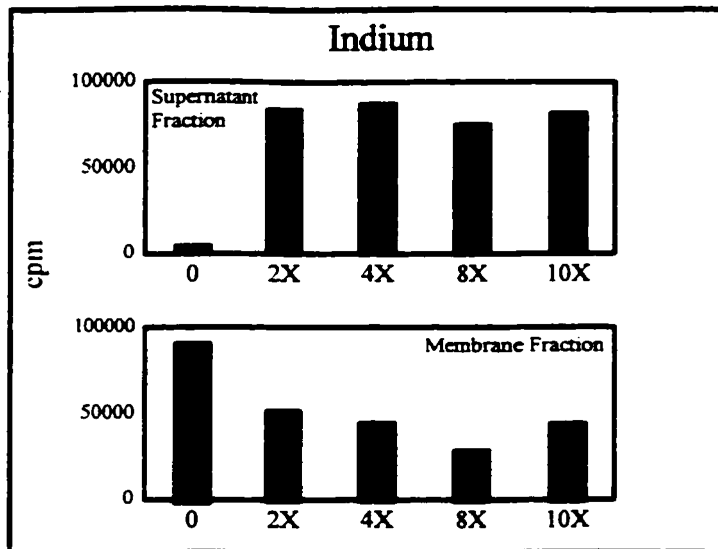
Disruption by repeated freeze-thawing (10 cycles) resulted in less than 10% of cytoplasmic marker (^{111}-Indium, Figure 6A), less than 3% of dense granule marker (^{14}C-serotonin, Figure 6B), and less than 2% of alpha granule marker (PF4, Figure 6C) in the platelet membrane fraction, respectively. In comparison, when platelets were ultracentrifuged alone (no disruption procedure), virtually all measurable cytoplasmic marker, dense granule marker and alpha granule marker were associated with the platelet membrane fraction (Figure 6). For all three markers, no further degree of platelet disruption was achieved with additional cycles of freeze-thawing (15 and 20 cycles) (data not shown). Each of these experiments were performed with a minimum of two normal donors.

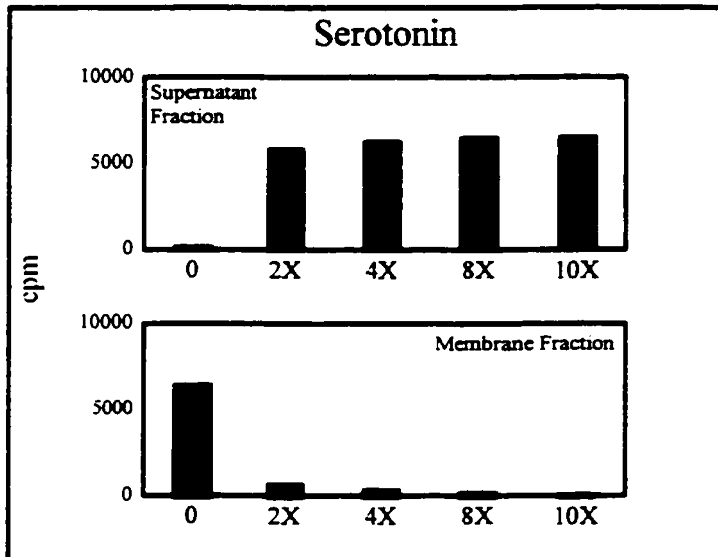
When membrane fractions of disrupted platelets were analyzed under a transmission electron microscope, morphologically distinct platelets were absent (Figure 7B). Additionally, structures resembling components of the normal intracellular platelet architecture, such as mitochondria or α-granules (Figure 7A), were not observed (Figure 7B). Together with platelet labeling studies, these observations supported the assumption that platelets were efficiently disrupted by repeated freeze-thawing.

Platelet-associated IgG measurements in platelet lysate (total PAIgG) (Figure 8A), platelet membrane fractions (Figure 8B) and platelet supernatant fractions (Figure 8C) were compared between normal adult controls (n=7), a group of well-characterized adult ITP patients (n=9), and a group of well-characterized adult patients with non-immune hematological disorders (thrombocytopenic, n=7; normal platelet count, n=7). The mean and standard deviation (mean, SD) for each patient and control group are summarized in Table 2. The mean total PAIgG values were significantly elevated in platelet lysates from thrombocytopenic patients including ITP patients (11.54, 5.19; $p=0.0002$) and patients with non-immune hematological disorders (7.67, 3.19; $p = 0.0058$) in comparison to normal controls (3.5, 0.82). Mean PAIgG values in platelet lysates from patients with normal platelet counts (4.07, 0.89; $p>0.05$) did not vary significantly from the control group (3.5, 0.82). In both the normal control group and all patient groups, the majority of platelet IgG was found to be associated with the platelet supernatant fraction (~95%). The amount of IgG associated with the membrane fraction expressed as a percentage of total IgG (membrane plus supernatant fractions) was as follows: 2.8% in normal controls, 7.0% in ITP patients, 2.9% in patients with non-immune thrombocytopenia and 2.9% in patients classified with non-immune hematological disorders and normal platelet counts.

Figure 6 Comparison of increasing degrees of platelet disruption using repeated freeze-thawing of labeled platelets.

Increasing degrees of freeze-thawing were compared, using platelets labeled with markers of intact external and intracellular membranes. Following ultracentrifugation alone (no disruption procedure), virtually all measurable cytoplasmic marker (^{111}-Indium) (A), dense granule marker (^{14}C-serotonin) (B) and alpha granule endogenous protein marker (PF4) (C), was associated with the platelet membrane fraction. Following platelet disruption by repeated freeze-thawing (10 cycles), less than 10% of cytoplasmic marker (A), less than 3% of dense granule marker (B), and less than 2% of alpha granule marker (C) were associated with the platelet membrane fraction. For all three markers, no further degree of platelet disruption was achieved with additional cycles of freezing and thawing (15 and 20 cycles) (data not shown).


A
Indium
cpm
100000
50000
0
Supernatant Fraction
0
2X
4X
8X
10X
100000
50000
0
Membrane Fraction
0
2X
4X
8X
10X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Serotonin
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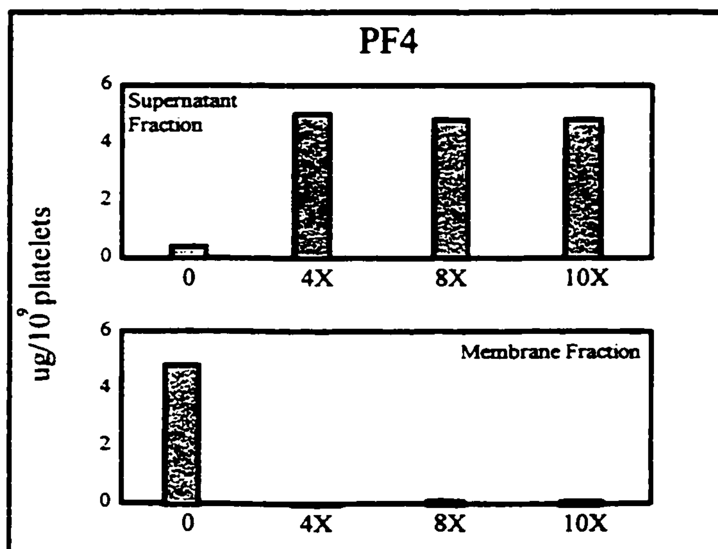

C
PF4
ug/10^9 platelets
6
4
2
0
Supernatant Fraction
0
4X
8X
10X
6
4
2
0
Membrane Fraction
0
4X
8X
10X

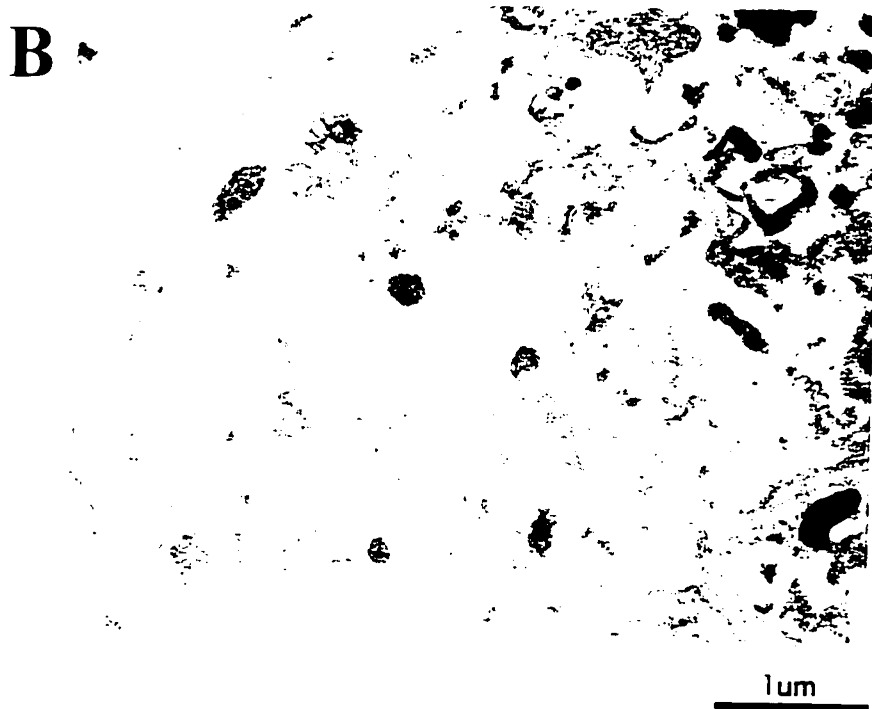
Figure 7 Electron micrograph of the membrane fraction of disrupted platelets.

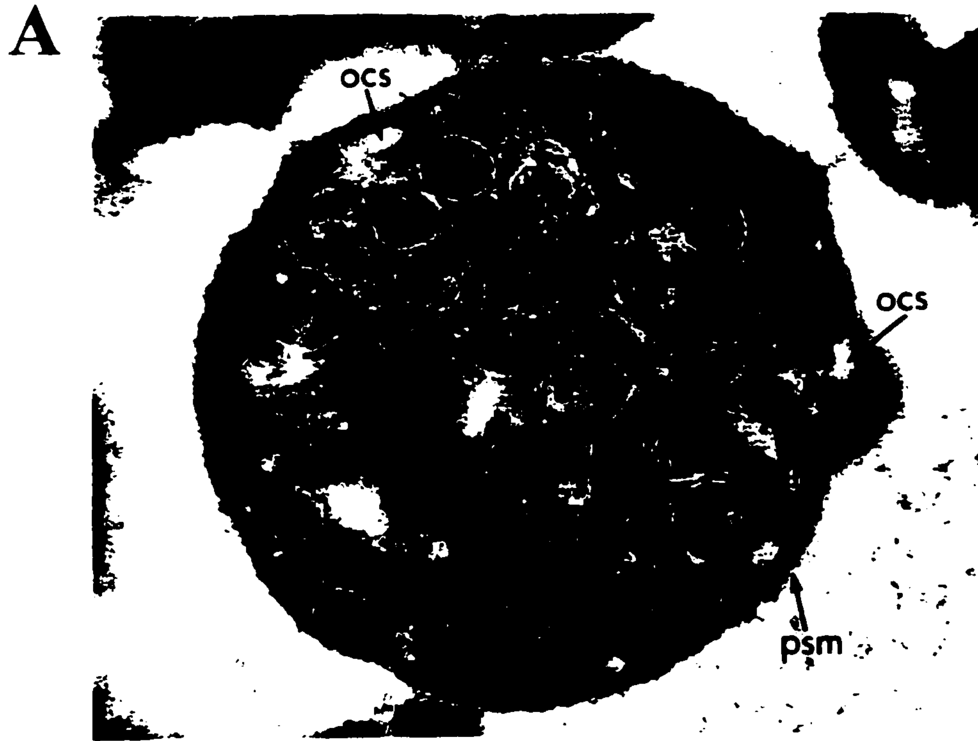
Control platelets (A), and the membrane fraction of disrupted platelets (B) were fixed in 1% glutaraldehyde and processed for transmission electron microscopy. Panel A illustrates the morphological appearance of a typical resting platelet. The intracellular platelet architecture includes, numerous secretory α-granules (a), mitochondria (m) and vacuolar structures of the open-cannalicular system (ocs). Panel B shows the morphological appearance of the membrane fraction of disrupted platelets showing an absence of normal platelet architecture with an abundance of membrane and organelle debris. It is not clear from the electron micrograph alone whether smaller platelet organelles such as granula remain intact.

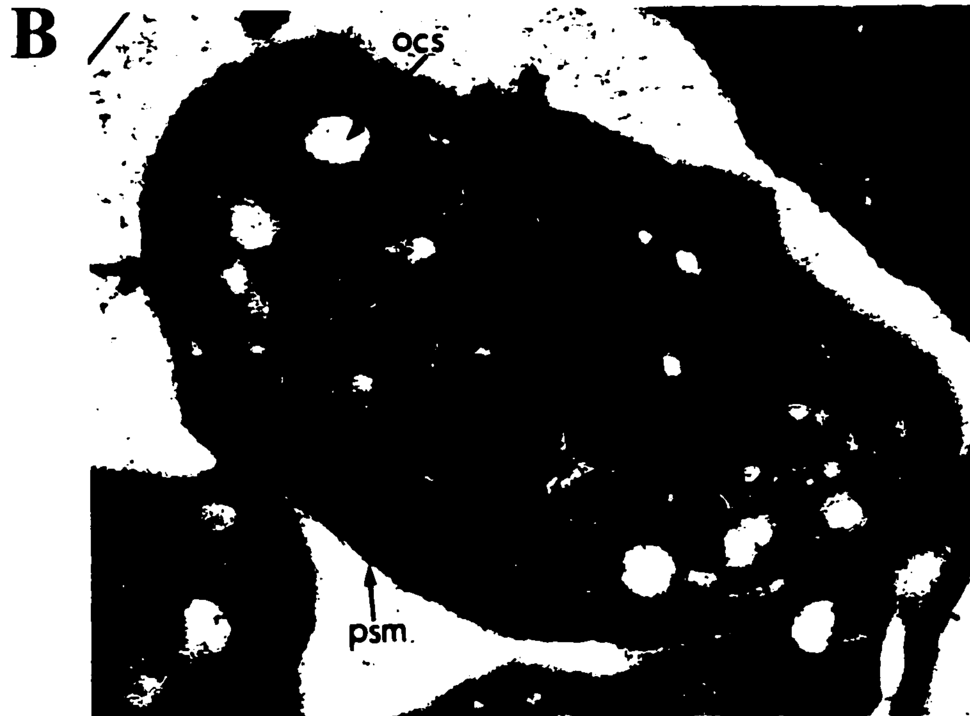
Original magnification: X 28, 000.

A

B




A
ocs
ocs
psm


B
ocs
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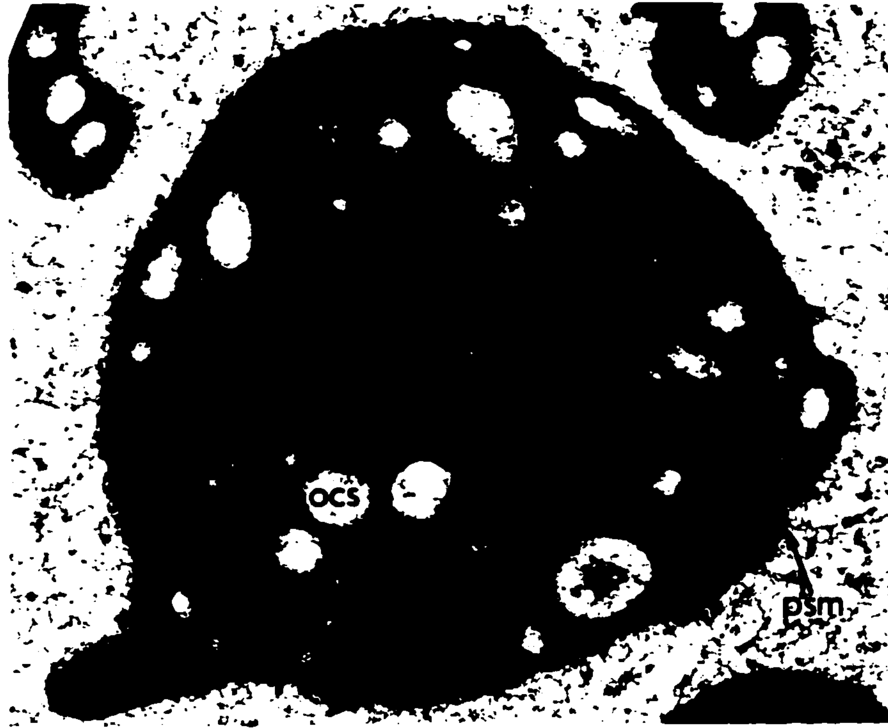
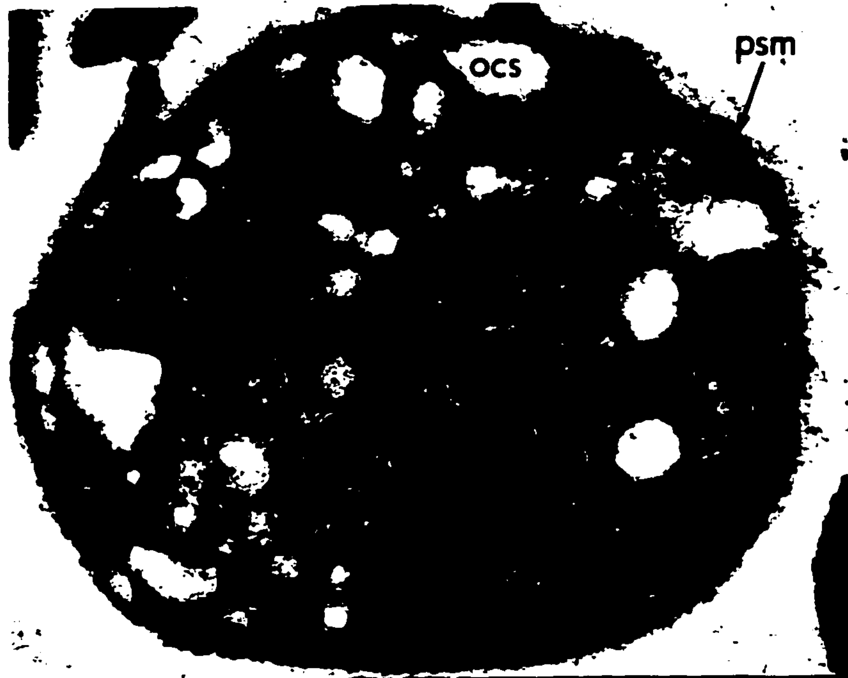
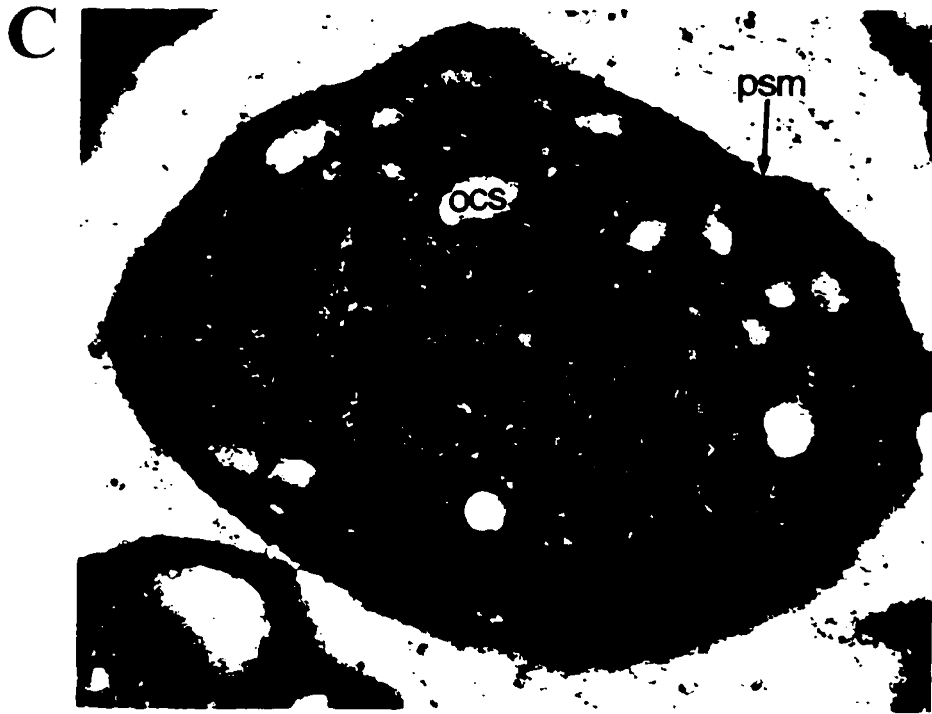
A



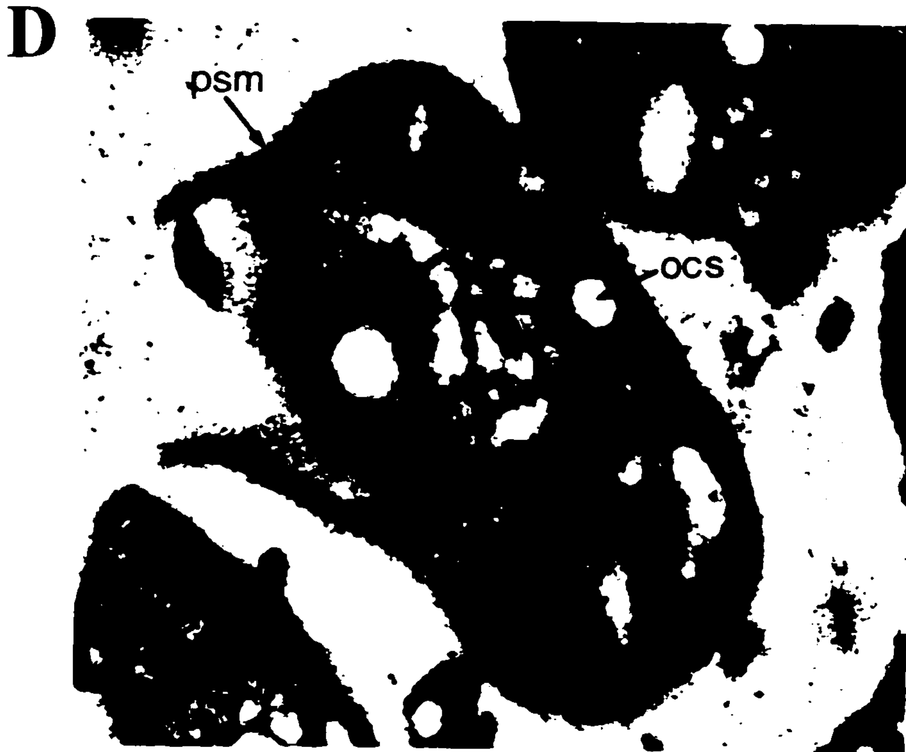
B



C



D



E

F

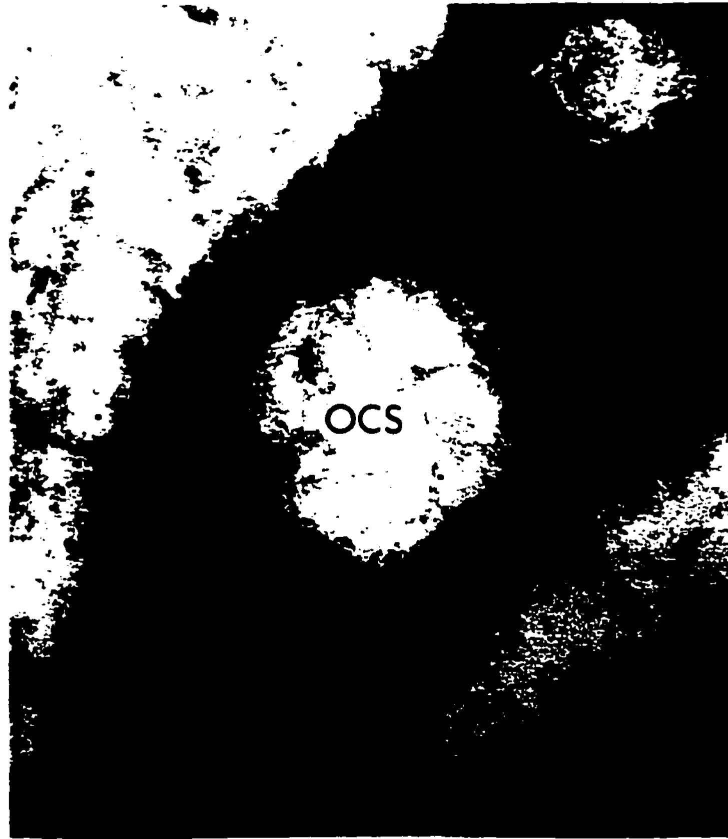
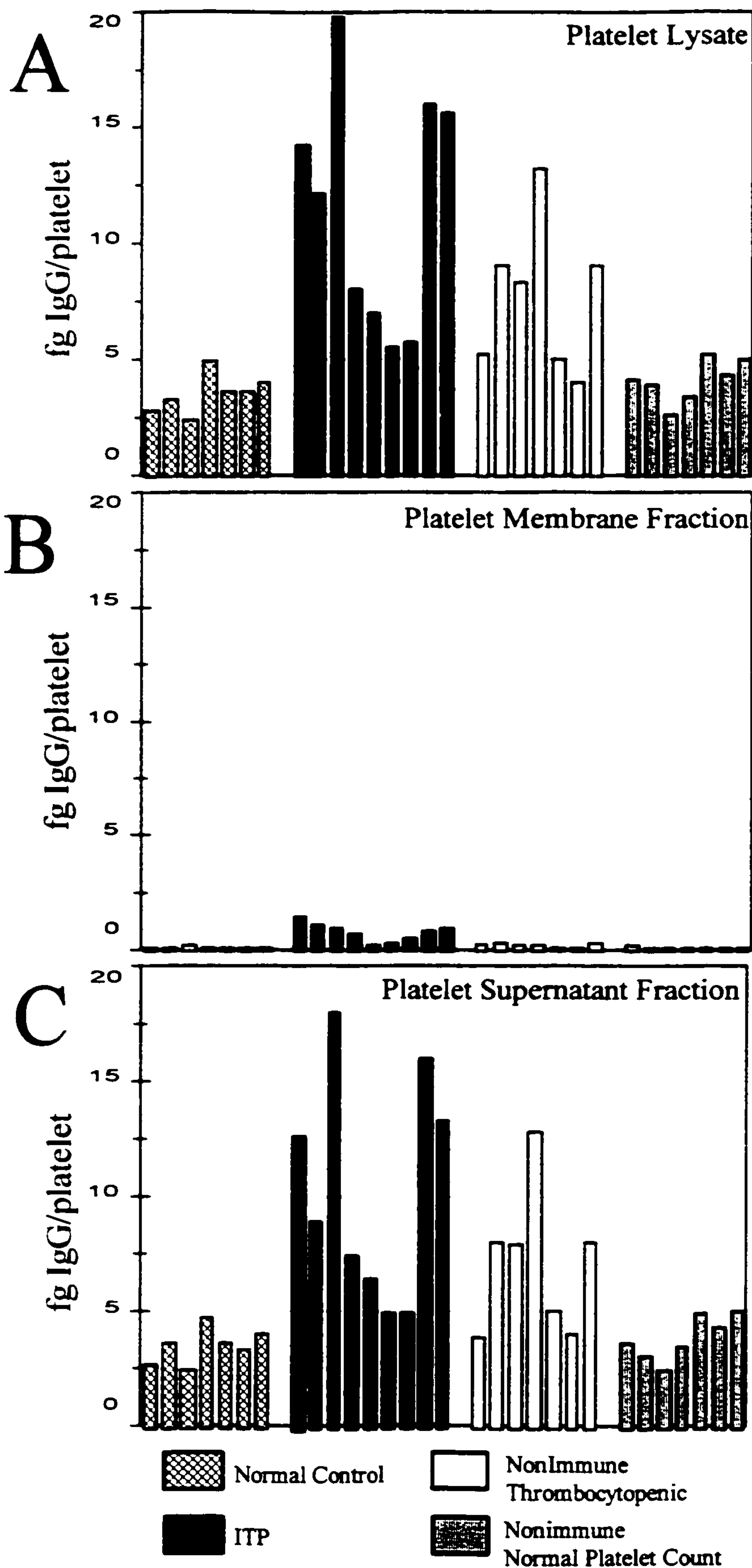


Figure 8 Measurement of PAIgG in platelet lysate, platelet membrane, and platelet supernatant fractions.

PAIgG measurements in platelet lysate (A), platelet membrane fractions (B), and platelet supernatant fractions (C), were compared between; normal adult controls (n=7), a group of well-characterized ITP patients (n=9) and a group of well-characterized adult patients with non-immune hematological disorders (thrombocytopenic, n=7; normal platelet count, n=7). In both normal controls and patients, the majority of PAIgG was associated with platelet supernatant fractions (~95%) with very little associated with platelet membrane fractions. The amount of IgG associated with platelet membrane fractions, expressed as a percentage of total IgG were as follows: 2.8% in normal controls, 7% in patients with ITP, 2.9% in patients with non-immune thrombocytopenia, 2.9% in non-immune patients with normal platelet counts. The mean PAIgG values and standard deviations for these measurements are summarized in Table 2.



A
Platelet Lysate
B
Platelet Membrane Fraction
C
Platelet Supernatant Fraction
fg IgG/platelet
20
15
10
5
0
Normal Control
ITP
NonImmune Thrombocytopenic
Nonimmune Normal Platelet Count

TABLE 2

Measurements of PAIgG in platelet lysate (total PAIgG), platelet membrane fractions and platelet supernatant fractions

	PLATELET COUNT (x 10^9/L)	PLATELET LYSATE (ug IgG/10^9 platelets)	PLATELET MEMBRANE FRACTION (ug IgG/10^9 platelets)	PLATELET SUPERNATANT FRACTION (ug IgG/10^9 platelets)
NORMAL CONTROLS (Normal Platelet Count, n=7)	291±24	3.50±0.82	0.10±0.05	3.46±0.78
ITP (Thrombocytopenic, n=9)	62±28	*&11.54±5.19*	*@0.77±0.40*	**10.27±4.88*
NONIMMUNE HEMATOLOGICAL DISORDERS (Thrombocytopenic, n=7)	61±34	*~7.67±3.19*	0.21±0.08	*^7.08±3.14*
NONIMMUNE HEMATOLOGICAL DISORDERS (Normal Platelet Count, n=7)	333±47	4.07±0.89	0.10±0.04	3.31±0.97
Defined Upper Limits of Normal (Mean + 2SD) (Based on Normal Controls)	>150	5.14	0.20	5.02

Data are reported as means ± standard deviations, n=number of samples. Italicized values are those which were significantly higher than the defined upper limits of normal: &p=0.00018, @p=0.00011, *p=0.00044, ~p=0.00583, ^p=0.01202.

3.1.3 Summary

In this study, platelet disruption techniques were utilized to measure PAIgG in patients with immune and non-immune thrombocytopenia. The purpose of these studies was to investigate the compartmentalization of PAIgG in platelets from thrombocytopenic patients by measuring the amount of membrane-associated PAIgG from the amount of soluble PAIgG (presumably, α-granular PAIgG). In this way, the gross subcellular distribution of PAIgG in thrombocytopenic patients was determined.

Using three markers of platelet membrane integrity, it was demonstrated that 10 cycles of freeze-thawing effectively disrupted external and intracellular platelet membranes. The absence of morphologically intact platelets in platelet membrane fractions was confirmed by electron microscopy. When PAIgG was measured in disrupted platelet fractions of normal controls (n=7), greater than 97'% of PAIgG was associated with the soluble fraction with less than 3% associated with the membrane fraction. In patients with non-immune hematological disorders having normal platelet counts (n=7), greater than 97% of PAIgG was associated with the soluble fraction with less than 3% associated with the membrane fraction. These results did not differ from the control group.

Measurement of PAIgG in platelet lysates from patients with ITP (n=9) and non-immune thrombocytopenia (n=7), demonstrated elevated total PAIgG values. When PAIgG was measured in disrupted platelet fractions of patients with non-immune thrombocytopenia, greater than 97% of PAIgG was associated with the soluble fraction with less than 3% associated with the membrane fraction. Despite elevated total PAIgG values, very little PAIgG was associated with the membrane

fraction. This amount was identical to that observed for normal controls and patients with normal platelet counts. In comparison, in patients with ITP (n=9), approximately 93% of platelet IgG was associated with the supernatant fraction, with 7% associated with the membrane fraction. This amount of membrane-associated IgG was significantly higher (p=0.0001) than that observed in normal controls or patients with non-immune thrombocytopenia.

These observations suggest that the gross subcellular distribution of PAIgG in patients with immune and non-immune thrombocytopenia is similar with the majority of PAIgG being associated with the soluble platelet fraction and very little PAIgG associated with the platelet membrane fraction. However, in patients with ITP, the amount of PAIgG on the platelet membrane fractions is significantly elevated in comparison to normal controls or to patients with non-immune thrombocytopenia. These results suggest, that elevated total PAIgG in patients with ITP may reflect elevations of both soluble and membrane-associated PAIgG, whereas elevated total PAIgG in patients with non-immune thrombocytopenia may reflect increased amounts of soluble IgG, alone.

Part 2 Immunolocalization of PAIgG at the light microscopic level

3.2 Introduction

In these studies, the subcellular distribution of PAIgG in platelets from patients with ITP and non-immune thrombocytopenia was determined using laser scanning confocal microscopy. The major technical advantage of the laser scanning confocal microscope is the ability to obtain images of a narrow plane of focus within a specimen (Pedley, 1997). In addition, computer digitization makes it possible to view a series of optical sections at incremental depths (Pedley, 1997). In this way, a stack of images can be assembled to form a three-dimensional reconstruction of the visualized specimen with greater image quality and resolution than would have been possible by simply viewing the specimen by conventional fluorescence microscopy (Pedley, 1997). To ensure the immobilization of IgG, platelets were chemically fixed prior to immunostaining. Using this procedure, it was possible to view both surface and internal IgG distribution patterns in non-permeabilized and permeabilized platelets, respectively. The purpose of these studies was to determine the immunolocalization of PAIgG, at the light microscopic level in patients with immune and non-immune thrombocytopenia.

3.2.1 Materials and methods

3.2.1.1 Patients and controls

In this investigation, platelets were studied from 19 adult patients with ITP, 23 adult patients with non-immune hematological disorders (thrombocytopenic, n=13; normal platelet count, n=10), and 18 healthy adult controls. In an antigen capture assay (outlined in section 2.2.4), platelet lysates from all patients with ITP demonstrated detectable anti-GPIIb/IIIa or anti-GPIb/IX antibodies, whereas

all non-immune patients and normal controls tested negative. Additionally, 5 patients diagnosed with ITP who were negative in an antigen capture assay (negative for anti-GPIIb/IIIa and/or anti-GPIb/IX antibody) were also studied.

3.2.1.2 Platelet collection and preparation

Whole blood was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1mM theophylline and prostaglandin E_1 (1 ug/ml) and centrifuged at 120 x g for 20 minutes to obtain platelet-rich plasma. Platelets were isolated by gel filtration on Sepharose CL 2B (Pharmacia Biotech, Baie d'Urfe, QC) columns pre-equilibrated with ACD/PBS buffer (pH 6.4) containing 1 mM theophylline and prostaglandin E_1 (1 ug/ml). Platelet fractions were collected into tubes containing 25 ug/ml apyrase, pooled, and counted. The gel-filtered platelets were incubated for 20 minutes at 22°C before use.

3.2.1.3 Determination of whether gel-filtration activates platelets

To determine whether gel-filtration activated platelets, the platelet activation marker, P-selectin (GMP-140), was measured in PRP (non-filtered platelets) and gel-filtered platelet samples, using flow cytometry. PRP and gel-filtered platelets were incubated with a FITC-conjugated anti-P-selectin monoclonal antibody (Cedarlane Laboratories, Guelph, ON) for 30 minutes at 37°C. As a positive control, PRP and gel-filtered platelets were incubated with the platelet activating peptide, TRAP (thrombin receptor activation peptide), both prior to and following platelet fixation. Platelets were analyzed using a Becton-Dickinson FACScan (Becton Dickinson, San Jose, CA). FITC-fluorescence was detected using a 530 nm band pass filter. Data for forward-angle scatter, side-angle scatter, and

FITC-fluorescence were obtained with gain settings in the logarithmic mode. For each sample, 5000 events were acquired. Platelets were identified and independently analyzed on the basis of their characteristic light scatter profiles on particle dot plots of forward light scatter versus right angle light scatter (Sims *et al*, 1988). Analysis of the fluorescence histograms was used to quantitate the platelet activation marker, P-selectin.

3.2.1.4 Platelet fixation and permeabilization

Platelets were fixed with an equal volume of 2% wt/vol paraformaldehyde in PBS (pH 7.5) for 1 hour at room temperature and quenched with an equal volume of 0.1 M glycine for 10 minutes. Triton X-100 permeabilization was adapted from the procedures described by Woods *et al* (1986) and Wencel-Drake *et al* (1987) and consisted of the addition of 0.5% vol/vol Triton X-100 to paraformaldehyde-fixed platelets, for 10 minutes at 22°C. Platelets were then washed once in platelet wash buffer and resuspended in 3% BSA/PBS (pH 7.4).

3.2.1.5 Antibodies

Antibodies used for immunofluorescent labeling studies included monoclonal (HB43) and polyclonal anti-human IgG (American Type Culture Collection, Rockville, MD and BioCAN Scientific, Mississauga, ON), monoclonal and polyclonal anti-human fibrinogen (BioCAN Scientific, Mississauga, ON), monoclonal and polyclonal anti-human albumin (BioCAN Scientific, Mississauga, ON and Serotec, Mississauga, ON) and several monoclonal antibodies developed in our laboratory including; monoclonal anti-human multimerin (JS-1) as described by Hayward *et al* (1993), monoclonal anti-human GPIIb/IIIa (Raj-1), and monoclonal anti-Ib (TW-1), as described by

Horsewood *et al* (1991). Secondary antibodies included Texas Red (TR) and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-sheep IgG (Jackson Immuno-Research Laboratories, West Grove, PA).

3.2.1.6 Immunofluorescent labeling of platelets

Immunofluorescent labeling experiments were performed by blocking paraformaldehyde-fixed platelets in 3% BSA/PBS (pH 7.4) with normal mouse or normal rabbit IgG (50 ug/ml) for 30 minutes at 37°C before primary antibody incubations. Primary antibodies included monoclonal antibodies against IgG, albumin, fibrinogen, and multimerin (a megakaryoycte synthesized α-granule protein) and several polyclonal antibodies, including sheep anti-albumin, rabbit anti-fibrinogen, and rabbit anti-human IgG. Routinely, primary antibodies were added at a concentration of 20 ug/ml. Platelets were incubated with primary antibody in blocking buffer for one hour at 37°C, washed and incubated with secondary Texas Red or FITC-conjugated antibody (1/100 dilution in blocking buffer) for one hour at 37°C. Platelets were washed and coverslip spreads of labeled platelets were dried in the dark before mounting onto glass slides using mounting media (Chemicon International Inc, Temecula, CA).

For double immunolabeling experiments, antibody incubations were performed sequentially, by using anti-IgG followed by the relevant fluorescent secondary antibody before labeling with the second primary antibody. Following an incubation period of one hour at 37°C, platelets were washed and coverslip spreads of labeled platelets were prepared and allowed to dry in the dark before mounting onto glass slides using mounting media.

Controls for the primary antibody included: no antibody, normal mouse IgG and normal rabbit serum. Controls also included platelets incubated with the primary antibody and relevant and irrelevant fluorescent secondary antibodies. Double-labeling experiments were performed in parallel with single-label experiments to validate the co-localization of primary antibodies following incubation with more than one fluorescent secondary antibody.

3.2.1.7 Confocal microscopy

Platelets were examined with an Axioplan Universal Microscopy (Carl Zeiss, Munich, Germany), using FITC, TR, and combination filters. Images were acquired with Northern Exposure Image Analysis Software (version 2.90, Empix Imaging Inc, Mississauga, ON), were separated into red and green channel images using Photoshop 3.0 (Adobe Systems Inc, Mountain View, CA) and imported into Micrografx Picture Publisher (version 7, Micrografx Inc, Richardson, TX).

Confocal scanning laser microscopy was performed with a Universal Confocal Laser Scan Research Microscopy System (Carl Zeiss), a 100X objective, and individual excitation lasers and filters for TR and FITC fluorochromes. Single-labeled preparations were used to set the levels for contrast and brightness in the double-labeled preparations to ensure that there was no crossover of the fluorochrome into the opposite channel.

3.2.1.8 Studies of whether permeabilization leads to loss of surface IgG

To determine whether the permeabilization of fixed platelets led to a loss of platelet surface marker, monoclonal anti-GPIIb/IIIa (Raj-1) antibody was radiolabeled with 1 mCi ^{125}I-sodium iodide

(specific activity of 192 mCi/mg protein) and incubated with gel-filtered platelets in Tyrode's buffer for 30 minutes at 37°C. Radiolabeled platelets were then fixed in paraformaldehyde, permeabilized or not permeabilized, and counted in an LKB 1275 minigamma counter (Fisher Scientific, Nepean, ON).

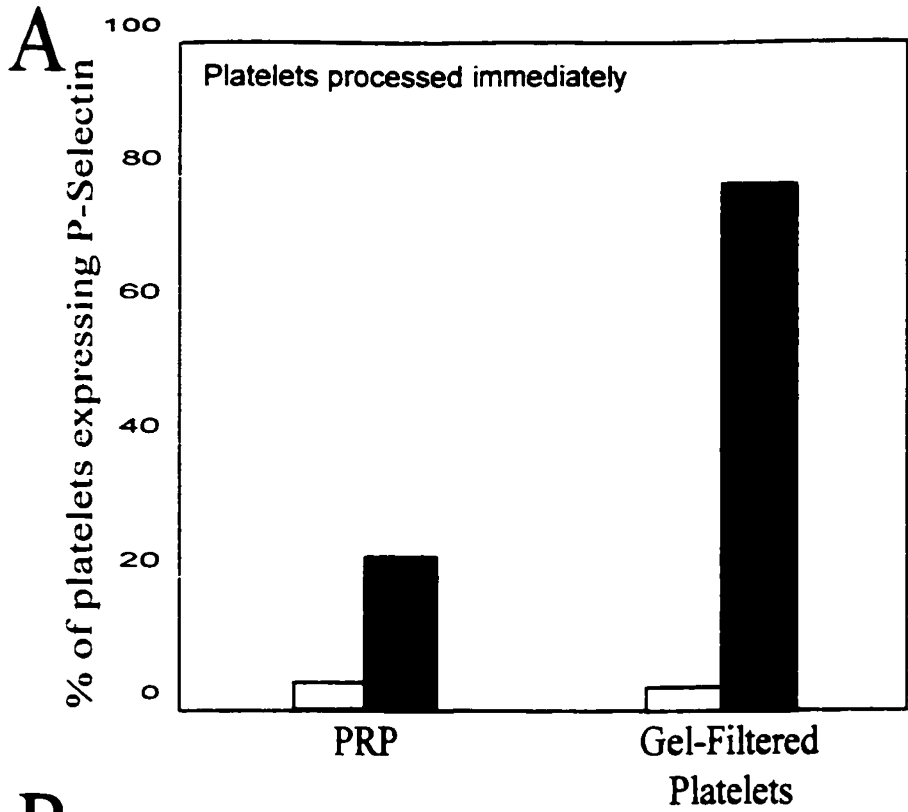
3.2.2 Results

In these studies, platelets were isolated from PRP by gel filtration. This method of platelet isolation has been reported as less activating than platelet isolation by differential centrifugation (Wencel-Drake *et al*, 1996; Simmons and Albrecht, 1996). When gel-filtered platelets were analyzed by flow cytometry for the expression of an activation marker (P-selectin), the percentage of platelets expressing this marker was less than 3% (Figure 9A). In comparison, the percentage of platelets expressing this marker in unfiltered, PRP was approximately 5% (Figure 9A). Following fixation with paraformaldehyde, the percentage of platelets expressing P-selectin did not increase from the original baseline level despite an overnight incubation or following deliberate incubation with an activating peptide (TRAP) (Figure 9B). These results demonstrated that the baseline level of platelet activation in PRP samples did not increase following gel filtration.

To determine whether permeabilization of fixed platelets resulted in loss of platelet surface marker, platelets were incubated with a radiolabeled anti-GPIIb/IIIa monoclonal antibody prior to fixation and permeabilization. These studies demonstrated that permeabilization of fixed platelets resulted in the loss of ~20% of the platelet surface radiolabel (Figure 10).

Figure 9 Analysis of P-selectin expression in gel-filtered platelets and unfiltered platelets (PRP), using flow cytometry.

To determine whether platelets were activated by gel filtration, PRP and gel-filtered platelets, were incubated with FITC-conjugated monoclonal anti-P-selectin (CD62) antibody, and analyzed by flow cytometry. As a positive control, platelets were deliberately activated with a thrombin receptor activation peptide (TRAP) (black bars). The percentage of platelets expressing the platelet-activation marker, P-selectin, in unfiltered platelets (PRP) and gel-filtered platelets were approximately 5%, and less than 3%, respectively (A). These results demonstrated that the baseline level of platelet activation in PRP samples, did not increase following gel filtration. As a positive control TRAP was added prior to fixation (A). Once platelets were fixed with paraformaldehyde, the percentage of platelets expressing P-selectin did not increase from the original baseline level despite an overnight incubation or addition of TRAP following fixation (B). In comparison, platelets which were not fixed and allowed to incubate overnight prior to incubation with anti-P-selectin, demonstrated an increase in the percentage of activated platelets for both gel-filtered and unfiltered platelets (PRP) (B).


A
Platelets processed immediately
% of platelets expressing P-Selectin
100
80
60
40
20
0
PRP
Gel-Filtered Platelets

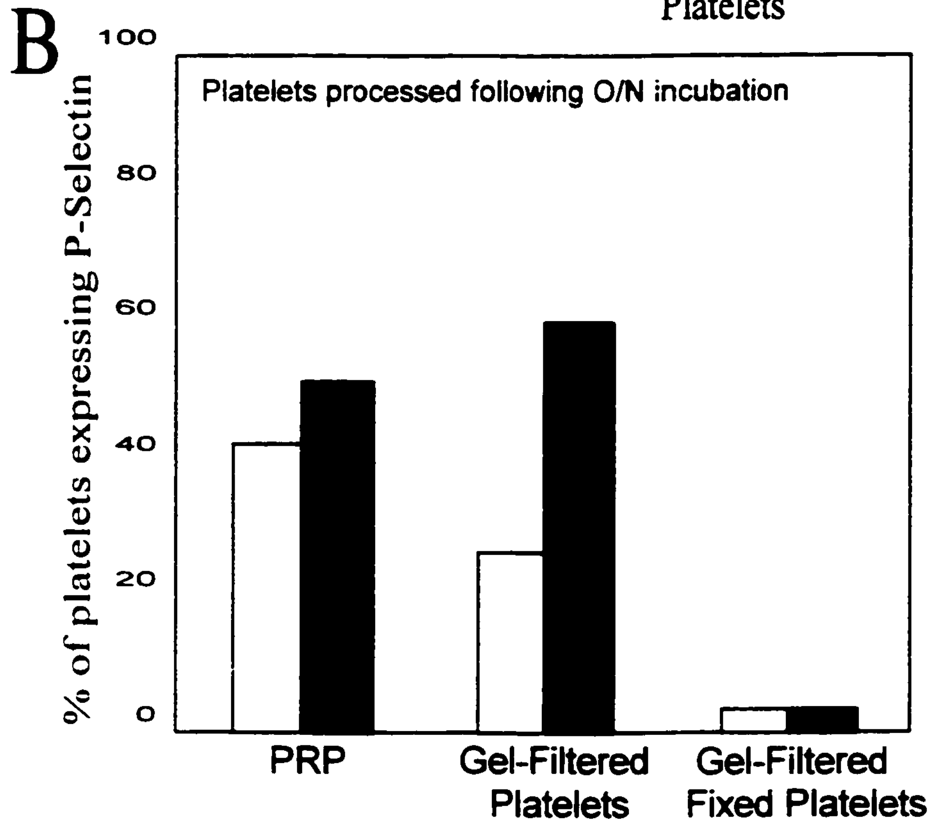
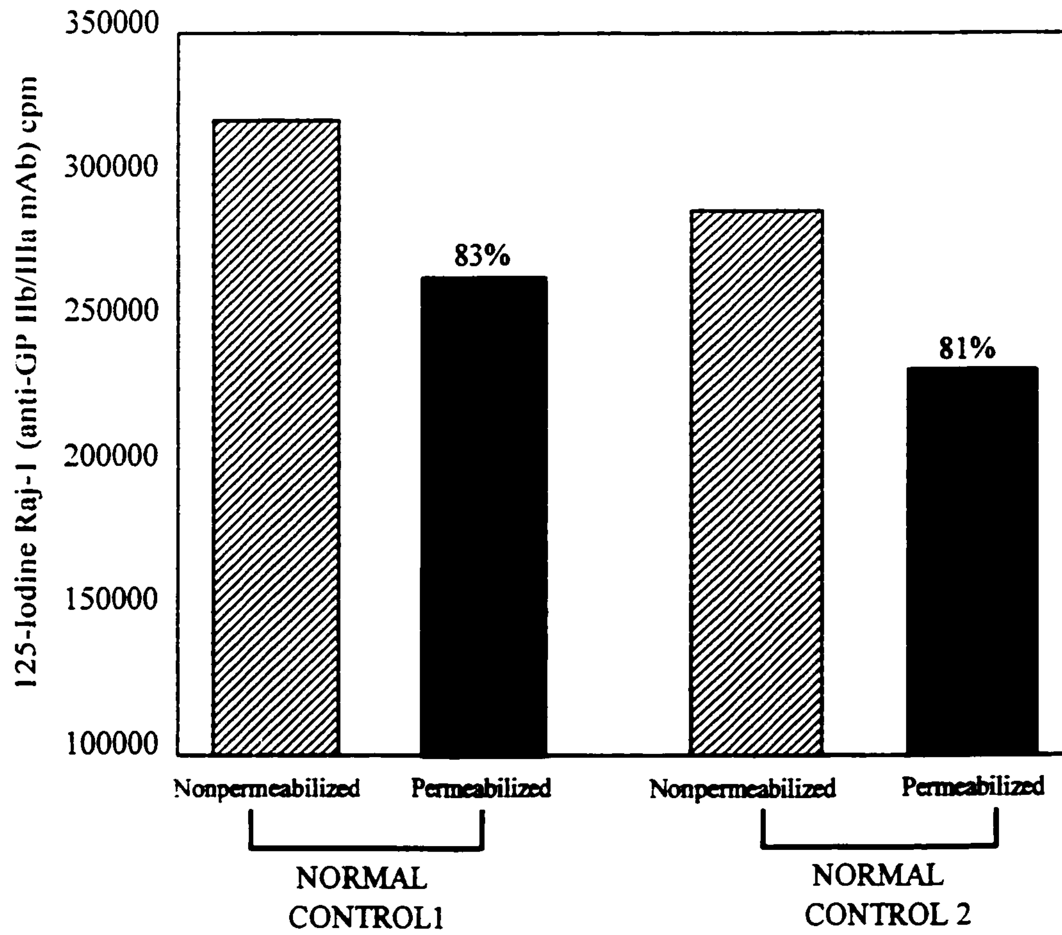

B
Platelets processed following O/N incubation
% of platelets expressing P-Selectin
100
80
60
40
20
0
PRP
Gel-Filtered Platelets
Gel-Filtered Fixed Platelets
RESTING
ACTIVATED (TRAP)

Figure 10 Permeabilization of fixed platelets and loss of platelet membrane.

To determine whether the permeabilization of fixed platelets led to a loss of platelet surface marker, platelets were incubated with a ^{125}I-radiolabeled monoclonal antibody directed against GPIIb/IIIa (Raj-1) before fixation and permeabilization. These studies demonstrated that the permeabilization of fixed platelets resulted in the loss of ~20% of the platelet surface marker.



350000
300000
250000
200000
150000
100000
125-Iodine Raj-1 (anti-GP IIb/IIIa mAb) cpm
83%
81%
Nonpermeabilized
Permeabilized
Nonpermeabilized
Permeabilized
NORMAL
CONTROL1
NORMAL
CONTROL 2

Control slides demonstrated no fluorescence when gel-filtered fixed human platelets were incubated with normal mouse IgG, normal rabbit serum, fluorescent secondary antibodies alone or with irrelevant primary and secondary antibodies (Figure 11).

When normal control platelets were labeled with antibodies directed against α-granule proteins, including fibrinogen, albumin, multimerin and IgG, a granular staining pattern was observed in permeabilized platelets (Figure 12). An overlay of serial platelet sections demonstrated that the staining was throughout the platelet (Figure 13) and that the granular staining pattern was similar for both endogenous (multimerin) and exogenous (IgG, fibrinogen and albumin) α-granule proteins. There was no difference in granular staining pattern between monoclonal and polyclonal primary antibodies, or Texas Red versus FITC conjugated secondary antibodies (not shown). Surface staining for alpha granular proteins (fibrinogen, albumin and multimerin) was not observed thus indicating their normal intracellular location (Figure 12).

When permeabilized platelets were stained for IgG, a granular pattern of staining was observed in all samples tested including, normal controls (n=18) (Figure 14), ITP patients with detectable anti-platelet antibody (n=19) (Figure 15), ITP patients with negative antigen capture results (n=5) (Figure 16), and patients with non-immune hematological disorders (n=23) (Figures 17, 18). There was no difference in the granular pattern of IgG staining between: (1) normal controls and patients, (2) patients with immune and non-immune thrombocytopenia, or (3) ITP patients with positive or negative antigen capture results.

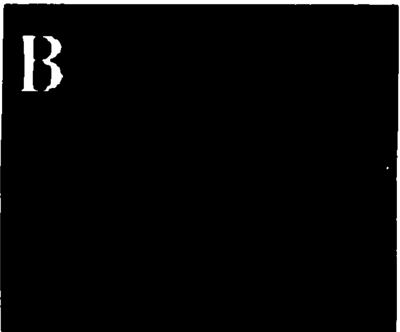
When non-permeabilized platelets were stained for IgG an absence of fluorescence was observed in normal controls (Figure 14) indicating an absence of IgG staining on the surface of platelets. In ITP patients with positive antigen capture results, a rim pattern of IgG staining in non-permeabilized platelets was consistently observed (Figure 15). In these patients, overlays of serial platelet sections demonstrated that IgG staining was on the surface of platelets forming a boundary which outlined the periphery of the platelet (Figure 19). This rim pattern of IgG staining was observed in 65-100% of platelets (100 platelets counted, n=15) and was similar to the rim pattern staining of membrane surface glycoproteins (GPIIb/IIIa and GPIb/IX) in normal platelets (Figure 20). In comparison, non-permeabilized platelets from ITP patients with negative antigen capture results demonstrated an absence of IgG staining on the surface of platelets (Figure 16). Patients with non-immune hematological disorders, whether thrombocytopenic (n=13) (Figure 17) or with normal platelet counts (n=10) (Figure 18) also demonstrated an absence of IgG staining on the surface of platelets.

Figure 11 Panel of control slides.

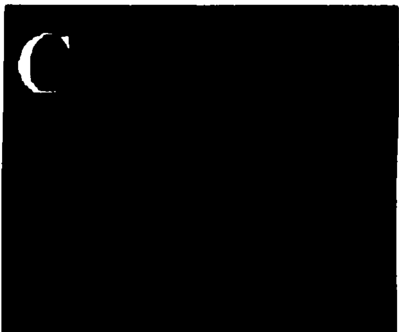
Control slides demonstrated no fluorescence when gel-filtered fixed human platelets (permeabilized and non-permeabilized) were incubated with normal mouse IgG (A), normal rabbit serum (B), fluorescent secondary antibodies alone [Texas Red-conjugated goat anti-rabbit IgG (C), FITC-conjugated goat anti-rabbit IgG (D)] or irrelevant primary and secondary antibodies [rabbit anti-human neurofilament (E), mouse anti-cholera toxin (F)].

PANEL OF CONTROL SLIDES

Mouse IgG



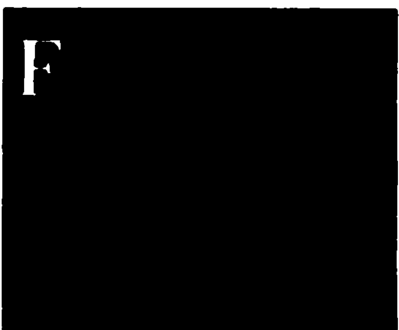
Rabbit Serum



Secondary Ab Alone
(TR-anti-rabbit)

Secondary Ab Alone
(FITC-anti-rabbit)

Irrelevant Primary Ab
(anti-neurofilament)

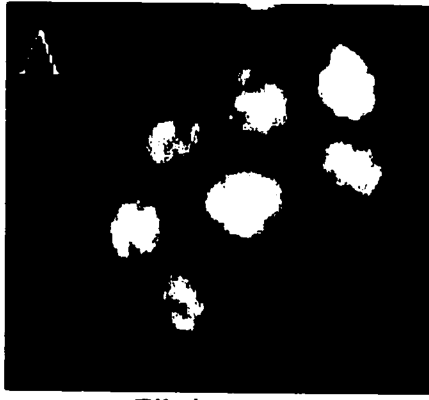


Irrelevant Primary Ab
(anti-cholera)

Figure 12 Immunolocalization of α-granule proteins in platelets.

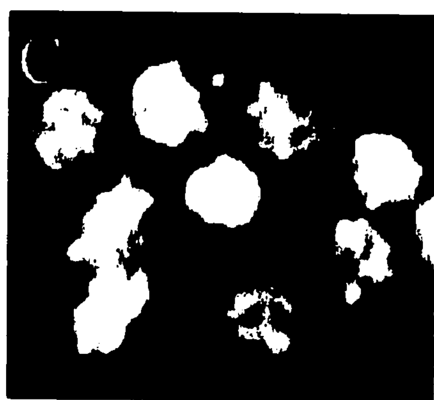
When normal control platelets were labeled with primary antibodies directed against α-granule proteins, including fibrinogen (A), albumin (B) and multimerin (C), and secondary Texas Red-conjugated antibodies, a granular staining pattern was observed in permeabilized platelets. An overlay of serial platelet sections demonstrated that the staining was throughout the platelet (Figure 14) and that the granular staining pattern was similar for both endogenous (multimerin) and exogenous (fibrinogen and albumin) α-granule proteins. There was no difference in granular staining pattern between monoclonal and polyclonal primary antibodies or Texas Red versus FITC-conjugated secondary antibodies (not shown). Surface staining for alpha granular proteins (fibrinogen, albumin and multimerin) was not observed in non-permeabilized platelets thus indicating their normal intracellular location (D, E, F).

PERMEABILIZED



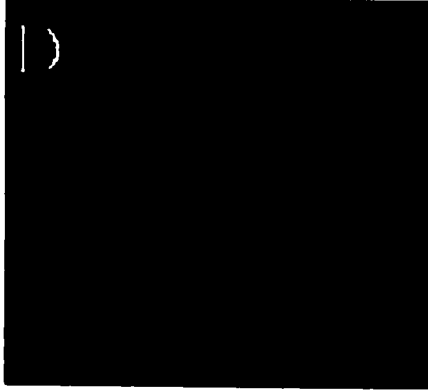
Fibrinogen

Albumin

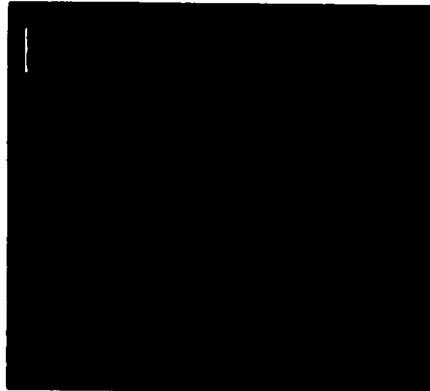


Multimerin

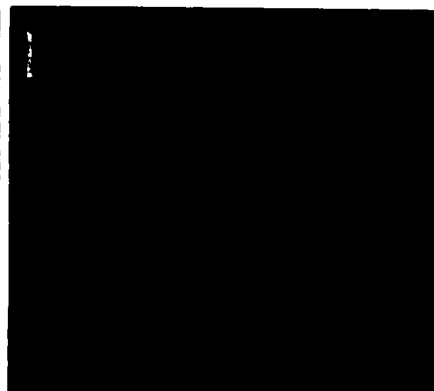
NONPERMEABILIZED



Fibrinogen



Albumin



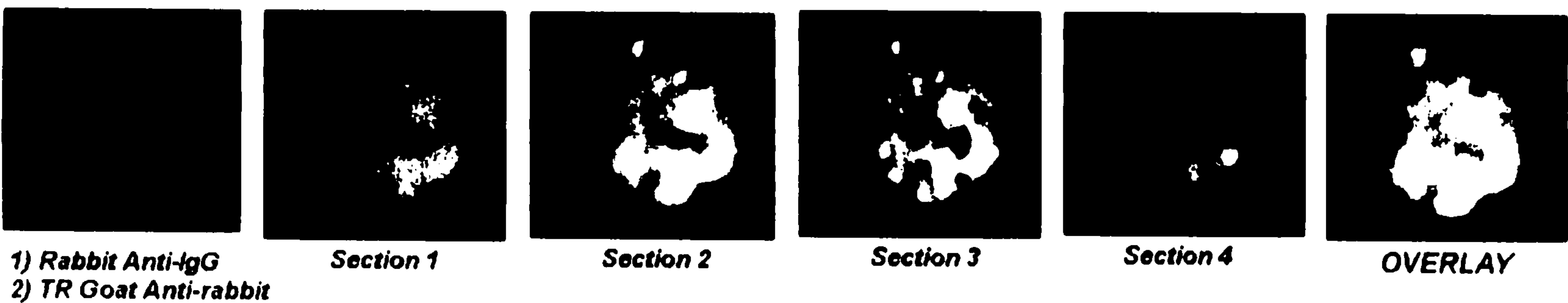
Multimerin

Figure 13 Granular staining pattern of IgG in permeabilized ITP platelets.

Two representative examples are shown of granular pattern staining in permeabilized ITP platelets as demonstrated by a series of optical sections acquired at incrementing depths through a platelet (A & B). A three-dimensional reconstruction of the visualized specimen (overlay) was formed by assembling the stack of optical images. These images demonstrate the staining of fixed permeabilized normal control platelets with rabbit anti-human IgG and Texas Red-conjugated goat anti-rabbit IgG antibody (the first image (in red) illustrates the two-dimensional view of platelet immunofluorescence using conventional confocal microscopy). This staining is typical of the granular staining pattern observed throughout the platelet for both endogenous and exogenous α-granule proteins.

Representative Examples of Granular Pattern Staining in Permeabilized Platelets Using Immunofluorescence and Confocal Microscopy

[Platelet Serial Sections - 600 nm Sections]



B

[Platelet Serial Sections - 600 nm Sections]

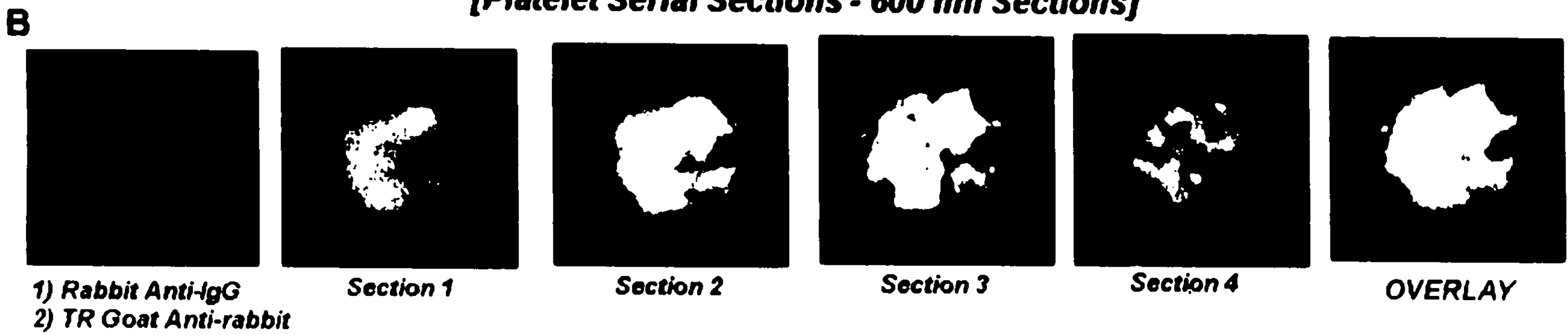
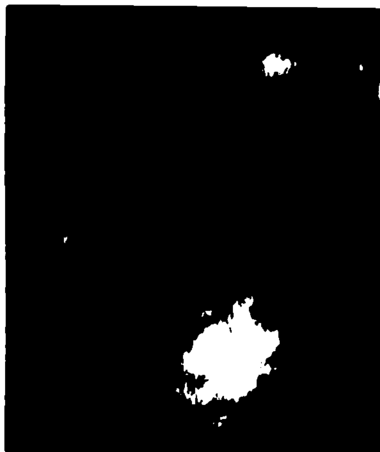


Figure 14 Immunolocalization of IgG in platelets from normal controls.

Fixed permeabilized and non-permeabilized platelets from normal controls (n=18) were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Platelets demonstrated an alpha granular staining pattern within platelets (permeabilized) and an absence of IgG immunofluorescence on the surface of platelets (non-permeabilized). Four representative controls are shown.

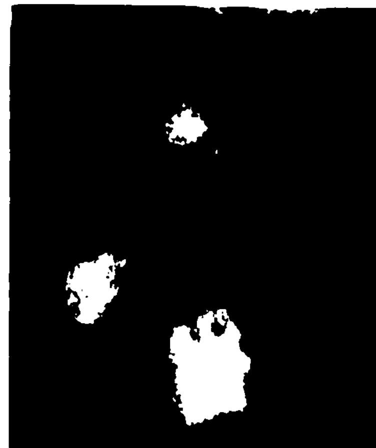
Permeabilized Platelets



Normal Control 1

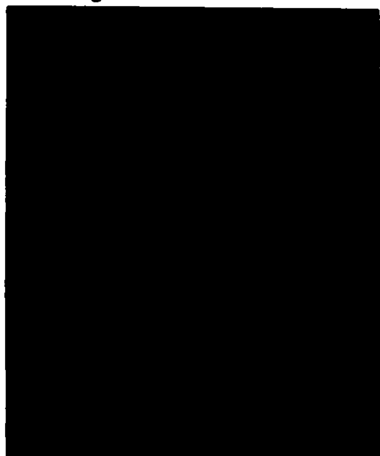
Normal Control 2

Normal Control 3

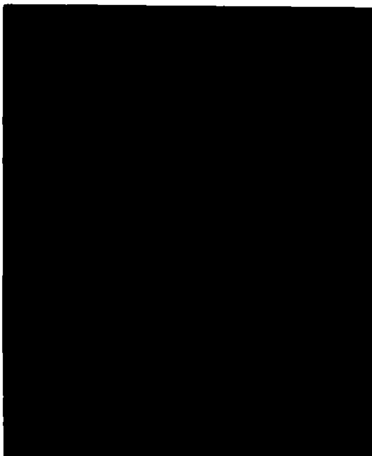


Normal Control 4

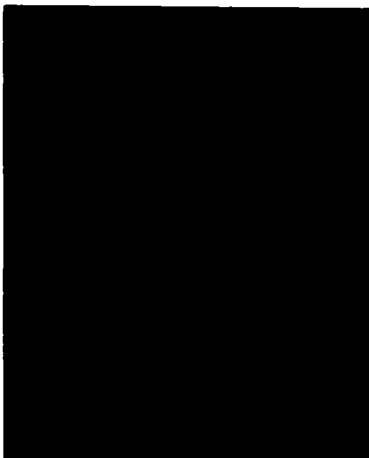
Nonpermeabilized Platelets



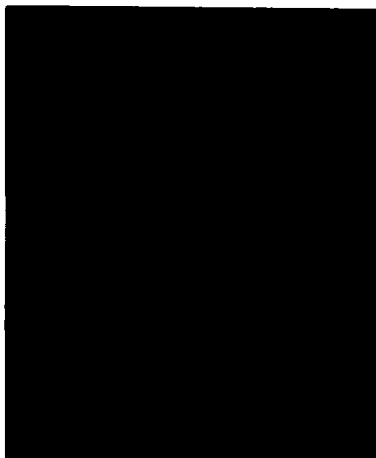
Normal Control 1



Normal Control 2



Normal Control 3

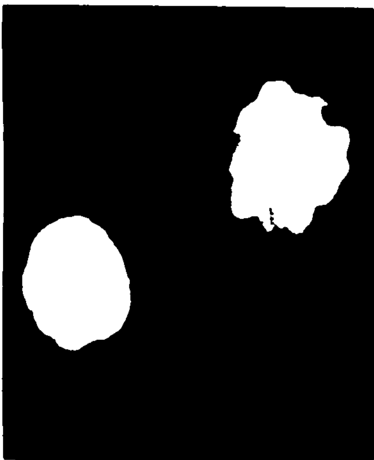


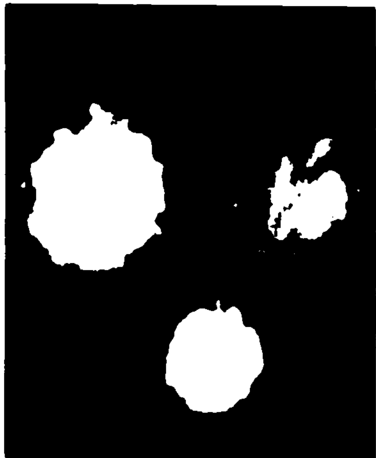
Normal Control 4

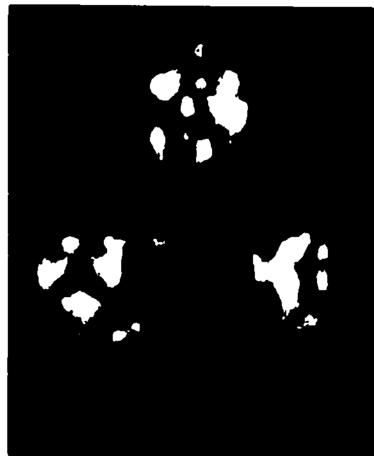
Figure 15 Immunolocalization of IgG in platelets from ITP patients with positive antigen capture results.

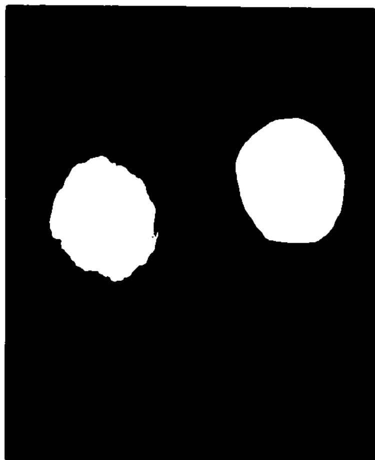
Fixed permeabilized and non-permeabilized platelets from normal controls (n=18) were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Platelets demonstrated an alpha granular staining pattern within platelets (permeabilized) and a rim pattern of staining on the surface of platelets (non-permeabilized). Four representative patients are shown.

Permeabilized Platelets


ITP Patient 1

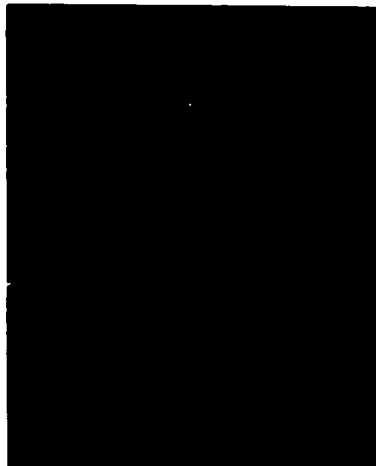

ITP Patient 2

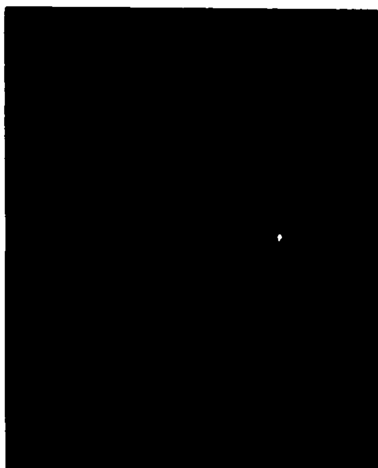

ITP Patient 3


ITP Patient 4

Nonpermeabilized Platelets

ITP Patient 1


ITP Patient 2


ITP Patient 3

ITP Patient 4

Figure 16 Immunolocalization of IgG in platelets from ITP patients with negative antigen capture results.

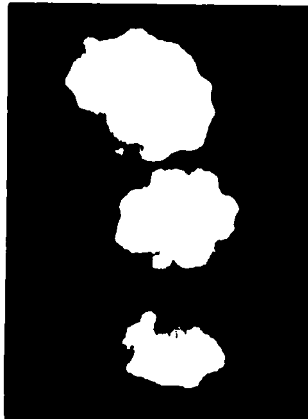
Fixed permeabilized and non-permeabilized platelets from ITP patients (n=5) were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Platelets demonstrated an alpha granular staining pattern within platelets (permeabilized) and an absence of IgG staining on the surface of platelets (non-permeabilized).

Permeabilized Platelets

ITP Patient 1
(negative AC result)

ITP Patient 2
(negative AC result)

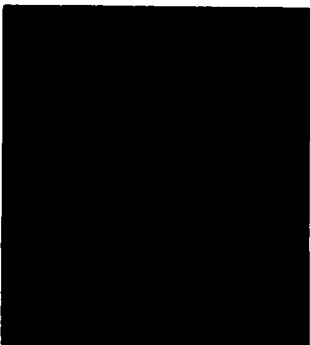
ITP Patient 3
(negative AC result)



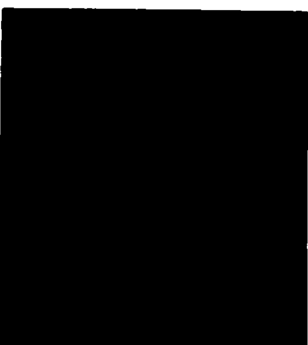
ITP Patient 4
(negative AC result)

ITP Patient 5
(negative AC result)

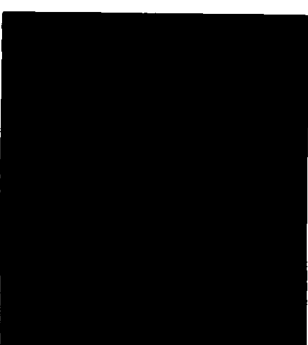
Nonpermeabilized Platelets



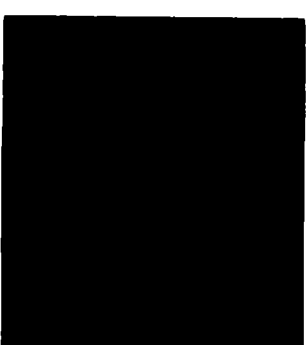
ITP Patient 1
(negative AC result)



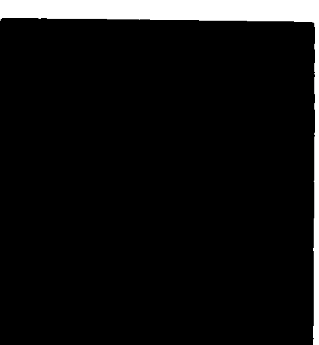
ITP Patient 2
(negative AC result)



ITP Patient 3
(negative AC result)



ITP Patient 4
(negative AC result)

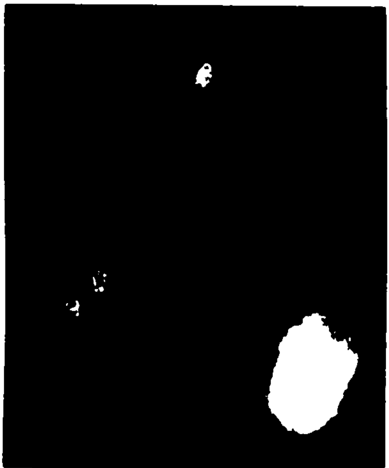


ITP Patient 5
(negative AC result)

Figure 17 Immunolocalization of IgG in platelets from patients with non-immune thrombocytopenia.

Fixed permeabilized and non-permeabilized platelets from patients with non-immune thrombocytopenia (n=13) were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Platelets demonstrated an alpha granular staining pattern within platelets (permeabilized), and an absence of IgG immunofluorescence on the surface of platelets (non-permeabilized). Four representative patients are shown.

Permeabilized Platelets



Nonimmune Thrombocytopenic Patient 1

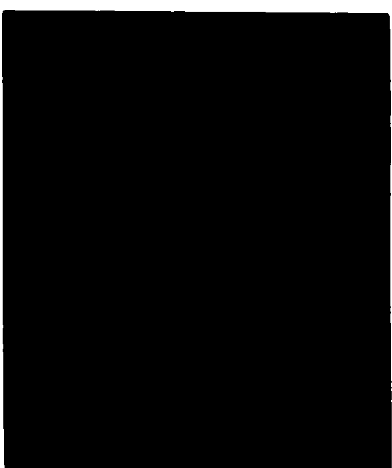
Nonimmune Thrombocytopenic Patient 2

Nonimmune Thrombocytopenic Patient 3

Nonimmune Thrombocytopenic Patient 4

Nonpermeabilized Platelets

Nonimmune Thrombocytopenic Patient 1



Nonimmune Thrombocytopenic Patient 2

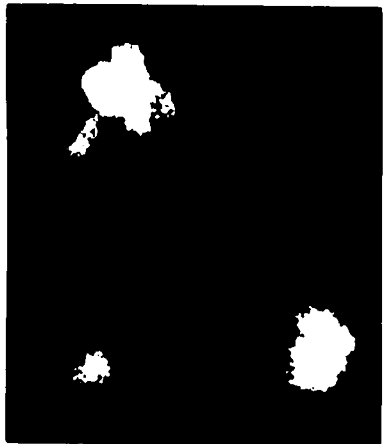
Nonimmune Thrombocytopenic Patient 3

Nonimmune Thrombocytopenic Patient 4

Figure 18 Immunolocalization of IgG in platelets from patients with non-immune hematological disorders and normal platelet counts.

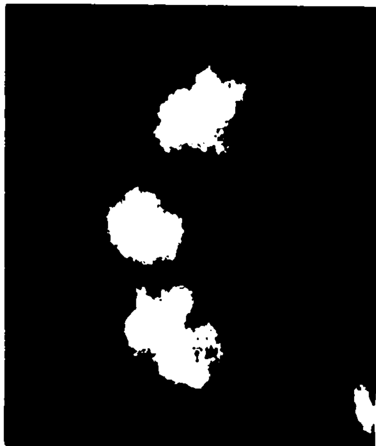
Fixed permeabilized and non-permeabilized platelets from patients with non-immune hematological disorders and normal platelet counts (n=10), were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Platelets demonstrated an alpha granular staining pattern within platelets (permeabilized) and an absence of IgG immunofluorescence on the surface of platelets (non-permeabilized). Four representative patients are shown.

Permeabilized Platelets



Nonimmune Hematological Disorder
Normal Platelet Count
Patient 1

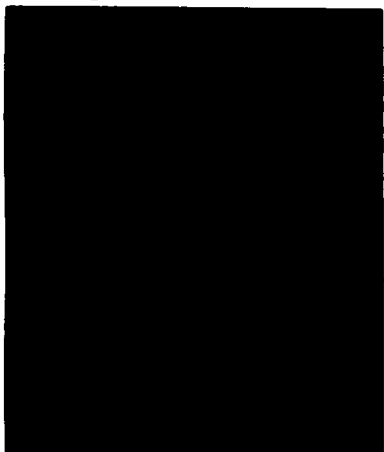
Nonimmune Hematological Disorder
Normal Platelet Count
Patient 2



Nonimmune Hematological Disorder
Normal Platelet Count
Patient 3

Nonimmune Hematological Disorder
Normal Platelet Count
Patient 4

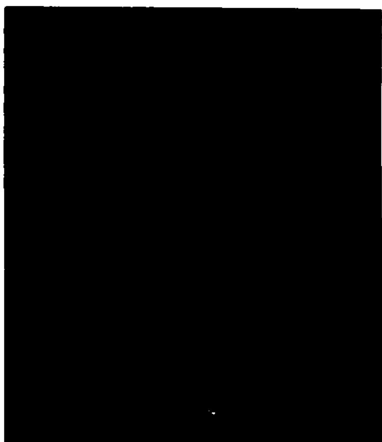
Nonpermeabilized Platelets



Nonimmune Hematological Disorder
Normal Platelet Count
Patient 1

Nonimmune Hematological Disorder
Normal Platelet Count
Patient 2

Nonimmune Hematological Disorder
Normal Platelet Count
Patient 3



Nonimmune Hematological Disorder
Normal Platelet Count
Patient 4

Figure 19 Rim staining pattern of IgG in non-permeabilized ITP platelets

Two representative examples of IgG rim pattern staining in non-permeabilized platelets from patients with ITP, as demonstrated by a series of optical sections acquired at incrementing depths through a platelet (A & B). A three-dimensional reconstruction of the visualized specimen (overlay) was formed by assembling the stack of optical images. This overlay of serial platelet sections demonstrated that IgG immunofluorescence was associated with the platelet surface. Images were obtained by incubating patient platelets with rabbit anti-human IgG and Texas Red-conjugated goat anti-rabbit IgG antibody (the first image (in red) illustrates the two-dimensional view of platelet immunofluorescence using conventional confocal microscopy).

Representative Examples of Rim Pattern Staining in Nonpermeabilized Platelets Using Immunofluorescence and Confocal Microscopy

[Serial Platelet Sections - 600 nm Sections]

[Serial Platelet Sections - 600 nm Sections]

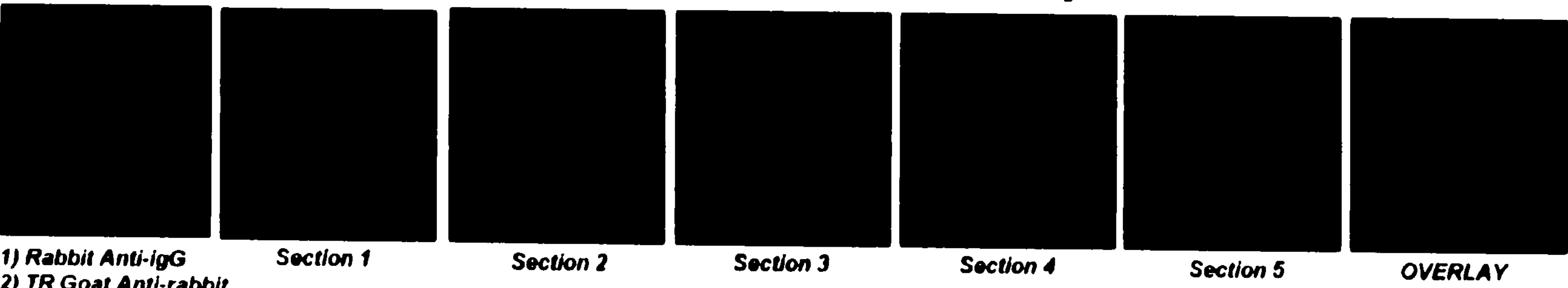


Figure 20 Immunolocalization of GPIIb/IIIa and GPIb/IX in non-permeabilized platelets.

Typical rim staining patterns of membrane glycoproteins in normal non-permeabilized platelets incubated with monoclonal anti-GPIIb/IIIa or anti-GPIb/IX antibodies, and Texas Red-conjugated goat anti-mouse IgG antibody. Overlays of serial platelet sections show that glycoprotein staining is on the platelet surface.

A

GPIIb/IIIa

B

GPIb/IX

3.2.3 Summary

In these studies, the subcellular distribution of PAIgG in platelets from patients with ITP and non-immune thromobocytopenia was determined using laser scanning confocal microscopy. To ensure the immobilization of IgG, platelets were chemically fixed prior to immunostaining and both surface (non-permeabilized platelets) and internal (permeabilized platelets) PAIgG distribution patterns were analysed.

Permeabilized platelets from normal controls demonstrated a granular pattern of IgG staining within platelets which was similar to that observed for other endogenous (multimerin) and exogenous (fibrinogen and albumin) α-granule proteins. This granular pattern of IgG staining was observed in platelets from all patients groups including: (1) patients with immune and non-immune thrombocytopenia, (2) patients with normal platelet counts and, (3) ITP patients with positive and negative antigen-capture results.

In normal controls, and non-immune patients with normal platelet counts, IgG staining on the surface of platelets (non-permeabilized platelets) was not observed. In comparison, ITP patients with detectable anti-platelet antibodies (positive antigen capture results) demonstrated a rim pattern of IgG staining on the platelet surface. This rim pattern of staining was similar to that observed for membrane glycoproteins on the surface of normal platelets. ITP patients with negative antigen capture results failed to demonstrate IgG staining on the surface of platelets. Additionally, patients with non-immune thrombocytopenia also demonstrated an absence of IgG staining on the surface of platelets. In these patients, despite the degree of thrombocytopenia, or elevated total PAIgG

values, IgG staining on the surface of platelets was not observed. These results suggest that surface patterns of IgG distribution differ in patients with immune and non-immune thrombocytopenia and in subsets of ITP patients with or without detectable anti-platelet antibody.

Part 3 Quantitation of IgG in permeabilized and non-permeabilized platelets, using flow cytometry

3.3 Introduction

In these studies, non-permeabilized and permeabilized platelets from patients with ITP and non-immune thrombocytopenia were analyzed using flow cytometry. The purpose of these studies was to quantitatively compare the amount of surface PAIgG (non-permeabilized platelets) and internal PAIgG (permeabilized platelets) in platelets from patients with immune and non-immune thrombocytopenia.

3.3.1 Materials and methods

3.3.1.1 Patients and controls

In this investigation, platelets were studied from 18 adult patients with ITP, 3 adult patients with ITP in remission (normal platelet counts), 27 adult patients with non-immune hematological disorders (thrombocytopenic, n=12; normal platelet count, n=15) and 24 healthy adult controls. In an antigen capture assay (outlined in section 2.2.4), platelet lysates from all patients with ITP (both active, n=18; and in remission, n=3), demonstrated detectable anti-GPIIb/IIIa or anti-GPIb/IX antibodies, whereas all non-immune patients and normal controls tested negative.

3.3.1.2 Platelet collection and preparation

Platelets were isolated by gel filtration as described in section 3.2.1.2, and fixed and permeabilized as described in section 3.2.1.4.

3.3.1.3 Antibodies

Antibodies used in flow cytometry studies included rabbit anti-human IgG (BioCAN Scientific, Mississauga, ON) and FITC-conjugated monoclonal anti-human IgG (CAG-2), as described by Horsewood *et al* (1991). Secondary antibodies included Texas Red (TR) and fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA).

3.3.1.4 Immunofluorescent labeling of platelets

Immunofluorescent labeling experiments were performed by blocking paraformaldehyde-fixed platelets in 3% BSA/PBS (pH 7.4) with normal mouse or normal rabbit IgG (50 ug/ml) for 30 minutes at 37°C before primary antibody incubations. Platelets were incubated with rabbit anti-human IgG primary antibody (20 ug/ml) for one hour at 37°C. Platelets were then washed and subsequently incubated with Texas Red- or FITC-conjugated secondary antibody (1/100 dilution in blocking buffer) for an additional hour at 37°C. Platelets were also incubated with a FITC-conjugated anti-human IgG (CAG-2) monoclonal antibody alone for one hour at 37°C.

Antibody controls included: no antibody, normal mouse IgG and normal rabbit serum. Controls also included platelets incubated with the primary antibody and relevant and irrelevant fluorescent secondary antibodies. In addition, the binding of fluorescent labels was compared between fixed and unfixed platelets from randomly selected patients and normal controls.

3.3.1.5 Flow cytometry

Platelets were identified using a Becton-Dickinson FACScan (Becton Dickinson, San Jose, CA). FITC-fluorescence was detected using a 530 nm band pass filter. Data for forward-angle scatter, side-angle scatter, and FITC-fluorescence were obtained with gain settings in the logarithmic mode. For each sample, 5000 events were acquired. Platelets were identified and independently analyzed on the basis of their characteristic light scatter profiles on particle dot plots of forward light scatter versus right angle light scatter (Sims *et al*, 1988). Analysis of the fluorescence histograms was used to quantitate PAIgG.

3.3.1.6 Statistical analysis

Statistical analyses were performed using the Corel Quattro Pro (Version 7) data analysis program. Inferences about PAIgG in permeabilized and non-permeabilized platelets for each patient group, was made by calculating the mean and standard deviation for each data set. The quantitative upper limit of normal for PAIgG in permeabilized and non-permeabilized platelets, was based on the data set of the healthy control group (mean + 2 standard deviations). The calculated mean for each data set was compared by using an analysis of variance (ANOVA, one-way) to determine whether differences among the population means were significant ($p<0.05$).

3.3.2 Results

In these studies, units of fluorescence, expressed on a logarithmic scale were used as a quantitative measure of platelet IgG in permeabilized and non-permeabilized platelets. When platelets from patients or normal controls were immunolabeled before fixation, or following fixation, no difference

in fluorescence values were observed (not shown). These results demonstrated that the fixation process itself did not result in increased binding of antibody to platelets.

In both patients and normal controls, platelet IgG was higher in permeabilized platelets than non-permeabilized platelets (Figure 21). In patients with ITP (n=18), mean PAIgG measurements (mean, SD) were significantly elevated both on the surface of platelets (120.33, 62.45) and within platelets (375.85, 303.91) compared to normal controls (n=24, non-permeabilized=34.33, 34.34; permeabilized=73.52, 31.13) (Table 3). In these patients, a detectable anti-GPIIb/IIIa antibody was demonstrated within platelet lysate samples in an antigen capture assay. Patients with ITP in remission (n=3) also demonstrated significantly higher IgG measurements both on the surface of platelets (109.88, 88.68) and within platelets (527, 186.79). Although these patients had normal platelet counts, anti-GPIIb/IIIa antibody was detected in platelet lysate samples in an antigen capture assay. Similar to thrombocytopenic ITP patients, patients with non-immune thrombocytopenia (n=12) had a significantly higher mean PAIgG measurement within platelets (173.23, 53.90), compared to normal controls. However, platelet IgG measurements were not elevated on the surface of platelets in these thrombocytopenic patients (36.67, 15.38). Finally, non-immune patients with normal platelet counts did not demonstrate elevated PAIgG measurements either on the surface of platelets or within platelets (n=15, non-permeabilized=41.46, 26.51; permeabilized=90.57, 34.42). The mean values and standard deviations for these measurements are summarized in Table 3.

Figure 21 Quantitative measure of PAIgG in permeabilized and non-permeabilized platelets from patients with immune and non-immune thrombocytopenia, using flow cytometry.

Flow cytometry was used to provide a quantitative measure of PAIgG in permeabilized and non-permeabilized platelets, based on the fluorescence of a FITC-conjugated anti-human IgG antibody. In patients with ITP (n=18), mean PAIgG measurements (mean, SD) were significantly elevated both on the surface of platelets (120.33, 62.45) and within platelets (375.85, 303.91), compared to normal controls (n=24, non-permeabilized=34.33, 34.34; permeabilized=73.52, 31.13). In these patients, a detectable anti-GPIIb/IIIa antibody was demonstrated within platelet lysate samples (not shown). Patients with ITP in remission (n=3), also demonstrated higher than normal IgG measurements on the surface of platelets (109.88, 88.68), and within platelets (527, 186.79). Although these three patients had normal platelet counts, anti-GPIIb/IIIa antibody was detected in their platelet lysate. Similar to thrombocytopenic ITP patients, patients with non-immune thrombocytopenia (n=12) had a significantly higher mean PAIgG measurement within platelets (173.23, 53.90), compared to normal controls. However, platelet IgG measurements were not elevated on the surface of platelets in these thrombocytopenic patients (36.67, 15.38). Finally, non-immune patients with normal platelet counts did not demonstrate elevated PAIgG measurements either on the surface of platelets or within platelets (n=15, non-permeabilized=41.46, 26.51; permeabilized=90.57, 34.42).

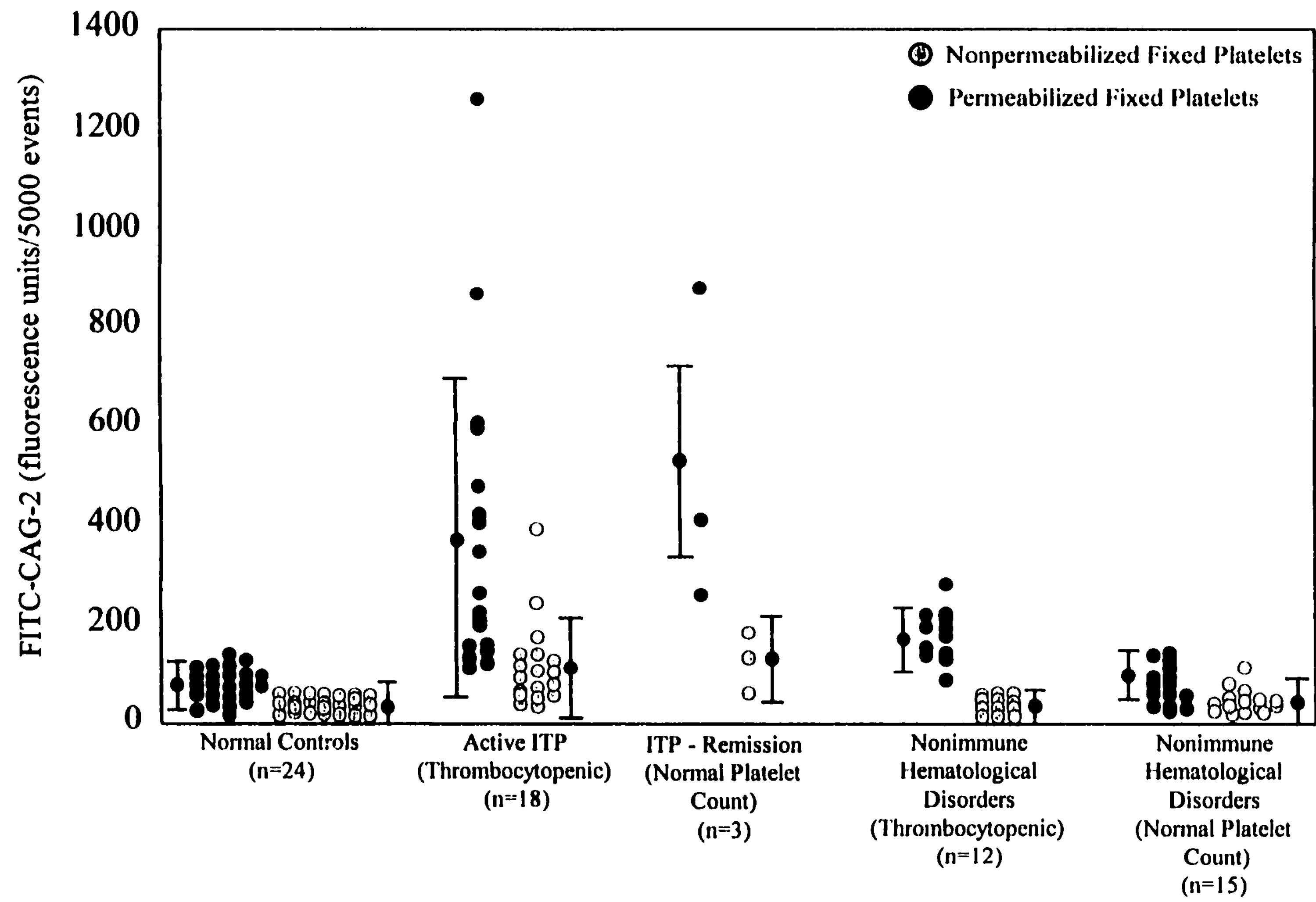

FITC-CAG-2 (fluorescence units/5000 events)
1400
1200
1000
800
600
400
200
0
Nonpermeabilized Fixed Platelets
Permeabilized Fixed Platelets
Normal Controls (n=24)
Active ITP (Thrombocytopenic) (n=18)
ITP - Remission (Normal Platelet Count) (n=3)
Nonimmune Hematological Disorders (Thrombocytopenic) (n=12)
Nonimmune Hematological Disorders (Normal Platelet Count) (n=15)

TABLE 3

Measurements of PAIgG (FITC-CAG-2) in permed and nonpermed platelets using flow cytometry.

	PLATELET COUNT (x 10^9/L)	NONPERMED PLATELETS (Surface PAIgG) (fluorescence units/5000 events)	PERMED PLATELETS (Internal & Surface PAIgG) (fluorescence units/5000 events)
NORMAL CONTROLS (Normal Platelet Count, n=24)	309±39	34.33±34.34	73.52±31.13
ITP (Thrombocytopenic, n=18)	53±36	*109.88±88.68	375.85±303.91
ITP-REMISSION (Normal Platelet Count, n=3)	240±81	@120.33±62.45	527±186.79
NONIMMUNE HEMATOLOGICAL DISORDERS (Thrombocytopenic, n=12)	79±46	36.67±15.38	^173.23±53.90
NONIMMUNE HEMATOLOGICAL DISORDERS (Normal Platelet Count, n=15)	309±198	41.46±26.51	90.57±34.42
Defined Upper Limits of Normal (Mean + 2 SD) (Based on Normal Controls)	>150	103.01	135.78

Data are reported as means ± standard deviation (SD), n=number of samples. Shaded boxes indicate values which were significantly higher than the defined upper limits of normal: *p=0.001985, ~p=0.013082, @p=0.047075, ^p=0.036421, &p=0.013482.

3.3.3 Summary

In these studies, units of fluorescence, expressed on a logarithmic scale, were used as a quantitative measure of platelet IgG in fixed, permeabilized and non-permeabilized platelets. Preliminary studies demonstrated that the fixation process itself, did not result in increased binding of antibody to platelets. In patients with ITP, either with active disease or in remission, PAIgG within platelets and on the platelet surface was significantly elevated ($p<0.04$) compared to the control group. In comparision, patients with non-immune hematological disorders did not demonstrate elevated surface PAIgG values. Despite elevated amounts of PAIgG within platelets of patients with non-immune thrombocytopenia, increased amounts of PAIgG on the platelet surface was not observed. These results suggest that PAIgG is elevated within platelets of patients with immune and non-immune thrombocytopenia, but is not elevated on the platelet surface of patients with non-immune thrombocytopenia.

Part 4 Ultrastructural evaluation of platelets, and immunolocalization of PAIgG, using electron microscopy

3.4 Introduction

In hematologic disorders, the contribution of electron microscopy as a diagnostic tool has varied. Ultrastructural studies have proven useful for the accurate identification and classification of certain lymphoid and hematopoietic malignancies, including, leukemias, lymphomas, histiocytoses and thymoma; however, the value of electron microscopy in the diagnosis and clinical management of platelet pathology is less clarified (Osborne, 1981; White, 1998).

Traditionally, the laboratory investigation of ITP has focused on the measurement of platelet autoantibodies. Alternatively, determination of the immunomorphologic characteristics of PAIgG in patients with ITP and non-immune thrombocytopenia, may prove useful for the understanding of why some IgG-sensitized platelets are cleared from the circulation (ITP) and others are not (non-immune thrombocytopenia). Using this approach, electron microscopic evaluation may contribute to the further understanding of a common autoimmune disorder and provide possible biological explanations for the increased levels of PAIgG in ITP and non-immune thrombocytopenic disorders. The purpose of these studies is to evaluate the platelet ultrastructure and subcellular distribution of PAIgG in patients with immune and non-immune thrombocytopenia.

3.4.1 Materials and methods

3.4.1.1 Patients and controls

In this investigation, platelets were studied from 6 well characterized adult patients with ITP, 6 well characterized adult patients with non-immune thrombocytopenia, and 6 normal adult controls. The average platelet count for the group of patients with ITP and non-immune thrombocytopenia was 64×10^9/L, and 68×10^9/L, respectively. In an antigen capture assay (outlined in section 2.2.4), platelet lysates from all patients with ITP, demonstrated detectable anti-GPIIb/IIIa antibodies, whereas all non-immune patients, and normal controls, tested negative.

3.4.1.2 Platelet collection and preparation of samples for electron microscopy

Platelets were isolated by gel filtration as described in section 3.2.1.2. Preliminary experiments were performed to compare preservation of platelet ultrastructure following fixation with 2% paraformaldehyde or 1% glutaraldehyde, and embedding in LR White resin or glycolmethacrylate. Based on these experiments, gel-filtered platelets were routinely fixed with an equal volume of 1% glutaraldehyde (in 0.1 mol/L phosphate buffer, pH 7.2) for one hour at room temperature. Samples were pelleted, embedded in glycolmethacrylate, and polymerized with UV light at 4°C.

3.4.1.3 Antibodies

Antibodies used for immunoelectron microscopy included: rabbit anti-human IgG Fc-specific and Fab-specific antibody (BioCAN Scientific, Mississauga, ON), rabbit anti-von Willebrand factor (DAKO, Carpenteria, CA), and goat anti-rabbit IgG coupled to 5nm or 10nm colloidal gold (Amersham, Oakville, ON).

3.4.1.4 Immunocytochemical procedures and electron microscopy

Thin sections of glycolmethacrylate embedded platelets were cut on a Reichert-Jung Ultracut ultramicrotome (Leica A.G., Vienna, Austria) and mounted onto formvar-coated grids. Platelet grids were blocked in 3% BSA/PBS (pH 7.4) with normal rabbit IgG (50 ug/ml) for one hour at room temperature before incubating overnight at 4°C with rabbit anti-human IgG Fc-specific antibody (40 ug/ml). Following several washes with filtered PBS (pH 7.4), platelet grids were incubated with goat anti-rabbit IgG labeled with colloidal gold (5 nm) (1/50) for two hours at room temperature and silver enhanced. Controls included platelet grids incubated with normal rabbit IgG, normal mouse IgG or secondary antibody alone. All sections were contrast-enhanced by uranyl acetate and lead citrate before examination under a JEOL 1200EX transmission electron microscope (Toyko, Japan).

3.4.1.5 Enumeration of gold particles

The distribution of colloidal gold particles on immunolabeled platelets was analyzed in *en face* electron micrographs printed at a final magnification of X12, 000. These electron micrographs were also used to enumerate the number of platelet organelles. The number of gold particles in 34 to 90 randomly chosen platelets were counted. The number of counted platelets varied depending on the degree of thrombocytopenia in each patient. A morphometric analyzer was used to determine platelet area (um^2) for each counted platelet. Only platelets with an area greater than 1 um^2 were counted. In each platelet, gold particles were counted on the platelet surface membrane, in channels of the open-cannalicular system and in α-granules. Gold particles which did not clearly localize to any of these regions were counted as 'other'. The total number of gold particles per platelet was determined

by summing the number of gold particles in each category, including those counted as 'other'. The counted platelets in these studies were also used for enumeration of platelet organelles.

3.4.1.6 Enumeration of platelet organelles

The average number of α-granules and mitochondria per platelet was determined by counting the number of each organelle in 34 to 90 randomly chosen platelets. Platelet α-granules and mitochondria were identified based on their characteristic morphological appearances (Cramer *et al*, 1985; Harrison and Cramer, 1992; Smith *et al*, 1997). Only platelets with an area greater than 1 um^2 were counted.

3.4.1.7 Statistical analysis

Statistical analyses were performed using the Corel Quattro Pro (Version 7) data analysis program. Inferences about number of gold particles, number of platelet organelles, and average platelet area, for each patient group, were made by calculating the mean and standard deviation for each data set. The quantitative upper limit of normal for each measurement was based on the data set of the healthy control group (mean + 2 standard deviations). The calculated mean for each data set was compared by using an analysis of variance (ANOVA, one-way) to determine whether differences among the population means were significant ($p<0.05$).

3.4.2 Results

Preliminary immunoelectron microscopy experiments were conducted to compare preservation of platelet ultrastructure following processing with different fixatives and embedding compounds. The

results from these studies demonstrated that platelet ultrastructure and PAIgG antigenicity, were best preserved when platelets were fixed in glutaraldehyde (verus paraformaldehyde) and embedded in glycolmethacrylate (versus LR White resin) (Figure 22).

Contrast-enhanced ultra-thin platelet sections demonstrate the subcellular ultrastructure of resting platelets (Figure 23). In these platelet sections, numerous organelles were observed throughout the platelet cytoplasm. The most prominent population of platelet organelles were the secretory α-granules. These spherical granules were 0.2- 0.35 um in diameter and enclosed by a limiting membrane. After α-granules, the next most commonly observed platelet organelles were mitochondria and the channels of the open-cannalicular system. Mitochondria were spherical to rod-shaped and morphologically identified by their highly folded inner membrane (cristae). In comparison, the open-cannalicular system appear as a tortuous system of clear elements (or vacuoles) dispersed throughout the platelet.

The ultrastructural morphology of ITP platelets (Figure 25), were similar to that observed for normal control platelets (Figure 23). No structural abnormalities were observed in ITP platelets. Under the transmission electron microscope, thin ITP platelet sections demonstrated a typical discoid form characteristic of resting platelets. As in normal platelets, the most predominant cytoplasmic organelle observed in ITP platelets was the platelet α-granule. These granules were morphologically heterogenous in shape, with some appearing spherical and others appearing elongated. In addition to α-granules, mitochondria and channels of the open-cannalicular system were observed throughout the cytoplasm. In platelet sections of ITP platelets, as in normal controls, large platelets or

megathrombocytes were occasionally present. The ultrastructural morphology of platelets from patients with non-immune thrombocytopenia (Figure 26) were similar to that observed for normal control platelets and ITP platelets. No structural abnormalities were observed in these platelets.

Immunogold localization of IgG on control grids, demonstrated an absence of gold labeling on platelet sections incubated with normal mouse IgG, normal rabbit IgG or blocking buffer alone (Figure 23). Platelet sections labeled with relevant primary antibody and irrelevant gold-conjugated secondary antibody demonstrated no gold labeling (Figure 23). Immunogold localization of IgG on thin platelet sections from normal controls (n=6), demonstrated labeling within platelet α-granules and to a lesser extent in unidentified structures resembling small vesicles (Figure 24). An absence of labeling was observed on the platelet surface membrane, within channels of the open-cannalicular system or in mitochondria (Figure 24). Random enumeration of gold particles in normal platelet sections, demonstrated an average of ~10 gold particles within α-granules, less than 1 gold particle on the platelet surface membrane, less than 1 gold particle associated with channels of the open-cannalicular system and 4 gold particles classified as 'other' per platelet section (Table 4). In these studies the category 'other' referred to gold particles within small vesicles (less than 0.1 um in diameter) or in platelet areas which could not be morphologically identified as α-granules, mitochondria, platelet surface membrane or channels of the open-cannicular system. Immunogold labeling of platelet sections from patients with ITP (n=6), demonstrated that the majority of gold particle labeling was within α-granules and small vesicles with some labeling of the platelet surface membrane and channels of the open-cannalicular system (Figure 25). Random enumeration of gold particles demonstrated an average of ~32 gold particles within α-granules, 3 gold particles on the

platelet surface membrane, 6 gold particles associated with channels of the open-cannalicular system and 18 gold particles classified as 'other', per platelet section (Table 4). The number of gold particles in these categories were significantly higher than that of normal controls (ANOVA, $p<1.2x10^{-5}$).

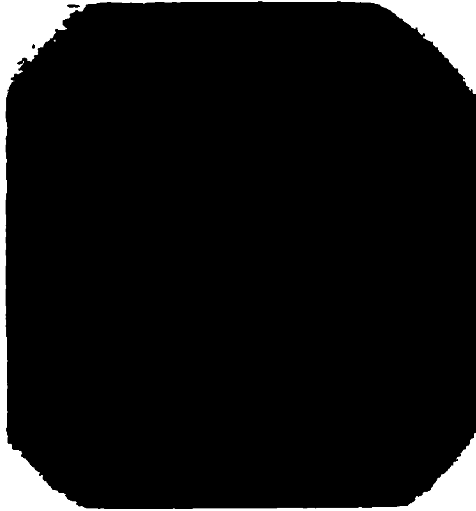
In patients with non-immune thrombocytopenia (n=6) immunogold localization of IgG demonstrated similar patterns of IgG distribution as that of normal controls with the majority of gold labeling observed within platelet α-granules and an absence of labeling on the platelet surface membrane or within channels of the open-cannalicular system (Figure 26). Random enumeration of gold particles demonstrated an average of ~20 gold particles within α-granules, less than 1 gold particle on the platelet surface membrane, less than 1 gold particle associated with channels of the open-cannalicular system and 6 gold particles classified as 'other' per platelet section (Table 4). In these patients the number of gold particles within α-granules was significantly higher than control values (ANOVA, $p=3x10^{-8}$) whereas, the number of gold particles on the platelet surface or within channels of the open-cannalicular system did not differ from that of normal controls.

To investigate whether increased numbers of gold particles in α-granules of thrombocytopenic patients was a reflection of increased platelet size or a greater number of α-granules per platelet, average platelet area (um^2), and average number of α-granules and mitochondria per platelet section, were determined. In normal controls, the average platelet area was 2.43 um^2 (n=342 counted platelets) (Table 5). In patients with ITP (n=327 counted platelets), and patients with non-immune thrombocytopenia (n=260 counted platelets), the average platelet areas were 2.67 um^2, and 2.48 um^2, respectively (Table 5). These values were not significantly different from control platelet sections

(ANOVA, $p>0.05$). Additionally, the number of α-granules and mitochondria per platelet section, did not differ between normal controls (9 α-granules and 3 mitochondria per platelet section) and thrombocytopenic patients (ITP, 11 α-granules and 3 mitochondria per platelet section; non-immune, 10 α-granules and 4 mitochondria per platelet section) (Table 5).

Figure 22 Comparison of preservation of platelet ultrastructure and antigenicity using different fixatives and embedding compounds.

The preservation of platelet ultrastructure and antigenicity of platelet proteins (IgG and von Willebrand factor) were compared using different fixatives and embedding compounds. Platelets were fixed with 2% paraformaldehyde and embedded in LR White resin (A, B), or fixed with 0.5% glutaraldehyde and embedded in LR White resin (C, D) or fixed with 0.5% glutaraldehyde and embedded in glycolmethacrylate (E, F). Platelet sections were immunolabeled with rabbit anti-von Willebrand factor (A, C, E) or rabbit anti-human IgG (B, D, F) and 10 nm gold-conjugated goat anti-rabbit IgG antibody. Platelet ultrastructure and antigenicity was judged to be best preserved when platelets were fixed with glutaraldehyde and embedded in glycolmethacrylate resin (E, F).


A

B

C

D

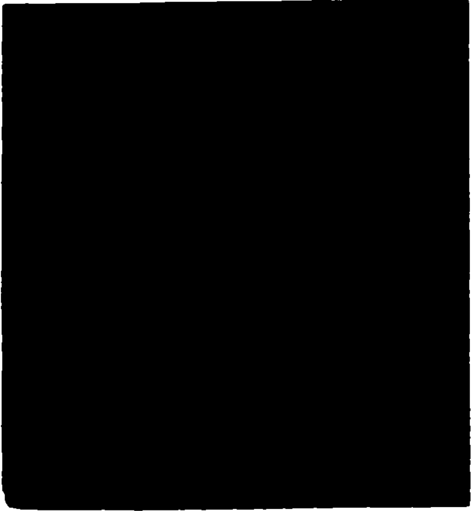
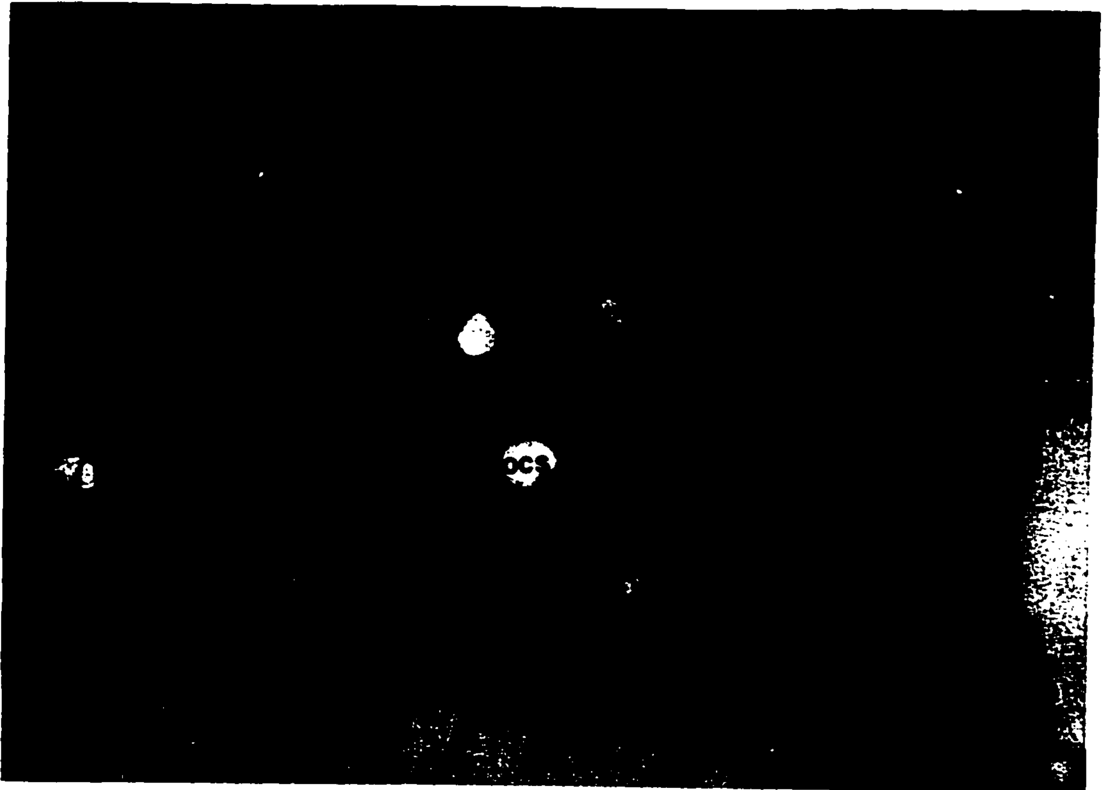
E
vWF

F
IgG

Figure 23 The ultrastructure of normal resting platelets.

Panels A-D illustrate the ultrastructure of normal resting platelets. Platelets were observed to be discoid in shape and contained several intracellular organelles enclosed by the platelet surface membrane (psm). The most prominent population of platelet organelles were secretory α-granules (a). These spherical granules were 0.2-0.35 um in diameter and enclosed by a limiting membrane. Other platelet organelles included mitochondria (m) and the open-cannalicular system (ocs). Mitochondria were spherical to rod-shaped and morphologically identified by their highly folded inner membrane (cristae). Channels of the open-cannalicular system appeared as vacuoles or vacuolar structures and were observed throughout the platelet. Control platelet sections demonstrated an absence of gold labeling when platelet sections were incubated with normal mouse IgG (A), normal rabbit IgG (B), or blocking buffer alone (C), and secondary gold-conjugated antibody. Platelet sections labeled with relevant primary antibody and irrelevant gold-conjugated secondary antibody also demonstrated no gold labeling (D).

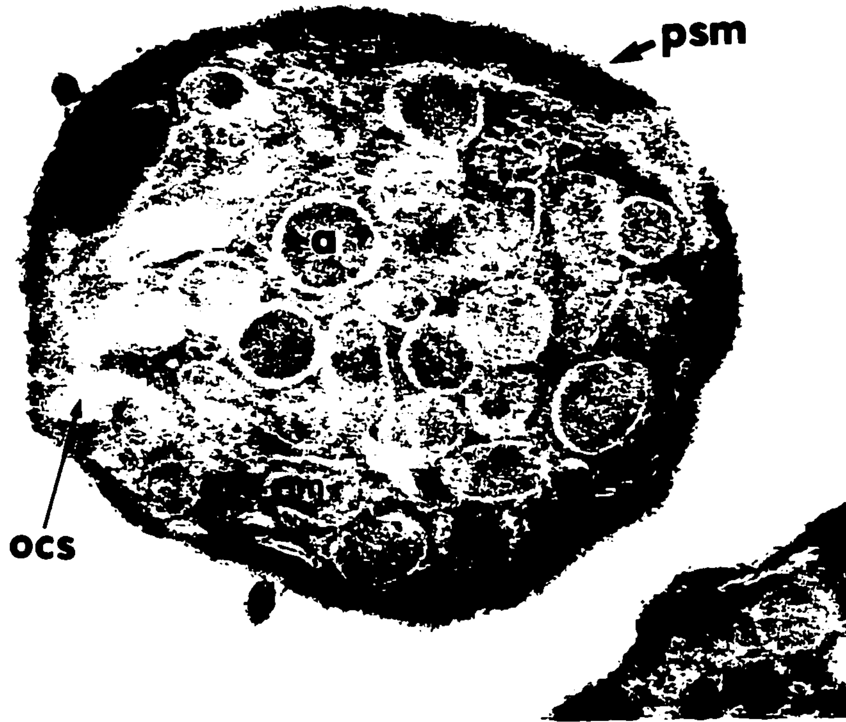
Original Magnification: X 40, 000 (A, B, C); X 37, 000 (D).

A



B

C



D

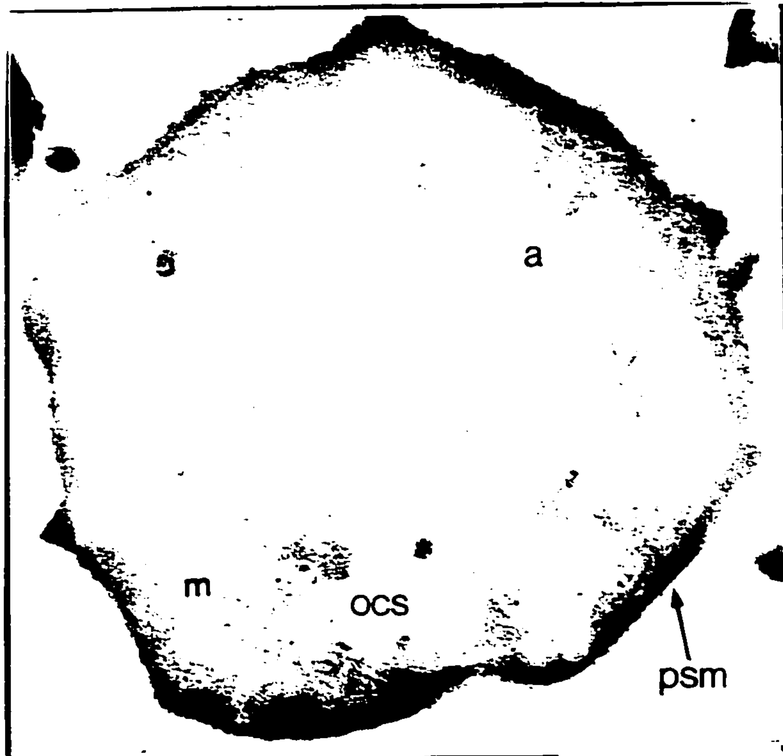


Figure 24 Immunogold localization of IgG in normal resting platelet sections.

Immunogold localization of IgG in normal resting platelet sections (A, B) demonstrated that the majority of labeling was associated with platelet α-granules (a) and to a lesser extent with small vesicles (less than 0.1 um in diameter) (see inset). An absence of labeling was observed on the platelet surface membrane (psm), within channels of the open-cannalicular system (ocs) or in mitochondria. Original Magnification: X 37, 500 (A); X 31, 200 (B).

A

B

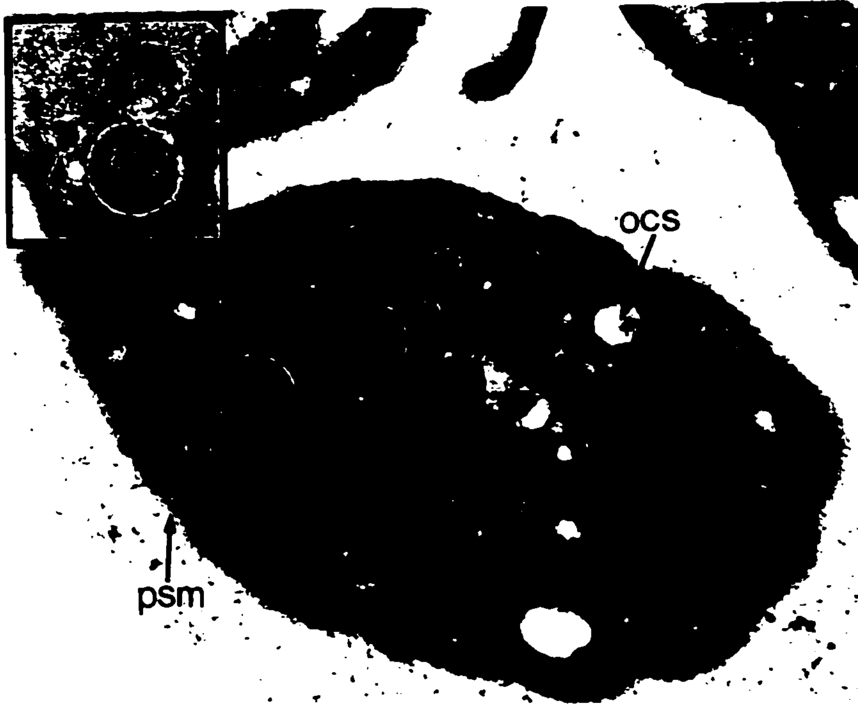


Figure 25 Ultrastructural morphology and immunogold localization of PAIgG in ITP platelets.

ITP platelets (A-E) were observed to have a typical discoid form, characteristic of normal resting platelets. The most predominant cytoplasmic organelles were α-granules (a). These granules were morphologically heterogenous, with some being spherical and others elongated. In addition to α-granules, mitochondria (m), and channels of the open-cannalicular system (ocs) were observed throughout the cytoplasm. In comparison to normal control platelets, no ultrastructural abnormalities were observed in ITP platelets. Immunogold localization of PAIgG, demonstrated the majority of labeling within platelet α-granules (a), or small vesicles (v) (less than 0.1 um in diameter) (A, B), with some labeling of the open-cannalicular system (ocs) and platelet surface membrane (psm) (C, D). Occasionally, a clustering of gold label was observed within a channel of the open-cannalicular system (E, F).

Original Magnification: X 35, 000 (A, B); X 37, 200 (C); X 34, 000 (D, E); X 112, 000 (F).

Figure 26 Ultrastructural morphology and immunogold localization of PAIgG in platelets from patients with non-immune thrombocytopenia.

Panels A and B illustrate the ultrastructural morphology of non-immune platelets. Platelets were observed to have a typical discoid form, characteristic of resting platelets. The most predominant cytoplasmic organelles were spherical or elongated α-granules (a). In addition to α-granules, mitochondria (m), small vesicles (v) and channels of the open-cannalicular system (ocs) were observed throughout the cytoplasm. No structural abnormalities of platelet architecture were observed. Immunogold localization of PAIgG, demonstrated the majority of gold labeling within platelet α-granules (a),with an absence of labeling on the platelet surface membrane (psm) or within channels of the open-cannalicular system (ocs). Original Magnification: X 26 400 (A); X 38 400 (B).

TABLE 4 - Average Number of Counted Gold Particles per Platelet Section

	# Counted Platelets	Ave. # Gold (Surface)	Ave. # Gold (OCS)	Ave. # Gold (Alpha Granules)	Ave. # Gold (Other)	TOTAL # GOLD	Ave. # Gold/um²
Normal Controls (n=6)	342	0.32±0.17	0.30±0.18	9.87±2.81	4.48±1.78	14.90±4.02	6.31±3.22
ITP (n=6)	327	3.34±0.15	5.7±2.11	32.34±11.58	18±5.83	58.35±9.54	22.41±8.15
Nonimmune Thrombocytopenia (n=6)	260	0.36±0.11	0.48±0.15	20.14±5.81	5.56±1.73	26.56±6.78	10.92±3.68

Data are reported as means ± standard deviation. Shaded boxes indicate values which were significantly higher than normal control values (ANOVA, $p<1.2 \times 10^{-5}$).

TABLE 5 - Average Number of Counted Alpha Granules and Mitochondria per Platelet Section

	# Counted Platelets	Ave. Platelet Area (um^2)	Ave. # Alpha Granules per Platelet	Ave. # Mitochondria per Platelet
Normal Controls (n=6)	342	2.43±1.07	8.99±5.81	2.86±1.18
ITP (n=6)	327	2.67±1.41	11.19±8.11	2.70±1.73
Nonimmune Thrombocytopenia (n=6)	260	2.48±1.12	10.08±6.56	3.60±1.47

Data are reported as means ± standard deviation. No patient values were significantly different from control platelets (ANOVA, $p>0.05$).

3.4.3 Summary

In these investigations, the platelet ultrastructure, and subcellular distribution of PAIgG, were evaluated in platelets, from patients with immune and non-immune thrombocytopenia. The results of these investigations were as follows. Firstly, apart from differences in numbers, ITP platelets and platelets from patients with non-immune thrombocytopenia, are morphologically identical to normal platelets, showing no evidence of structural abnormalities. Secondly, the vast majority of IgG in ITP platelets and non-immune platelets, is found within the α-granules. In ITP platelets, additional smaller amounts are associated with the platelet surface membrane and channels of the open-cannalicular system. Labeling of these structures was not observed in normal controls or patients with non-immune thrombocytopenia. Thirdly, the increased immuno-gold labeling of IgG in ITP platelets, and within α-granules of platelets from patients with non-immune thrombocytopenia, is not a reflection of increased platelet size, or increased numbers of storage granules per platelet. These results suggest that the subcellular distribution of PAIgG in patients with ITP and non-immune thrombocytopenia are different, but the mechanism by which IgG accumulates within platelets, may be similar.

Part of these studies have been published in Seminars in Hematology.

THE USE OF ELECTRON MICROSCOPY IN THE INVESTIGATION OF THE ULTRASTRUCTURAL MORPHOLOGY OF ITP PLATELETS

Mary Hughes, Kathryn Webert and John G. Kelton. SEMINARS IN HEMATOLOGY (2000) 37:1-8.

CHAPTER 4

The subcellular distribution of IgG in platelets following *in vitro* incubations with purified immune and non-immune IgG

4.1 Introduction

Currently, there is no adequate biological explanation for the increased amounts of PAIgG on both the platelet surface and within the platelet of patients with immune and non-immune thrombocytopenia. One proposed hypothesis suggests that plasma proteins may accumulate in platelets by a process of receptor-mediated or fluid-phase endocytosis. This hypothesis is based on numerous studies demonstrating that platelets are capable of sequestering a wide variety of particulate and soluble material within channels of the surface-connected canalicular system (Movat *et al*, 1965; White, 1968; Zucker-Franklin, 1981) and the more recent evidence that platelets have the ability to internalize and incorporate exogenous proteins directly into secretory α-granules (Handagama *et al*, 1987, 1989). The purpose of the present studies was to investigate possible pathways of IgG trafficking in platelets by determining the immunolocalization of IgG in normal platelets following incubation with anti-platelet antibody or non-immune IgG. Using immuno-gold labeling, it was possible to quantitate the amount of IgG within various platelet organelles over time. In this way possible pathways of internalization of immune versus non-immune IgG were compared.

4.2 Materials and methods

4.2.1 Platelet collection and preparation

Platelets from a normal type O donor, were isolated by gel-filtration as outlined in section 3.2.1.2 and resuspended in Tyrode's buffer (pH 7.4).

4.2.2 Antibodies

Antibodies used for laser scanning confocal microscopy and immunoelectron microscopy included rabbit anti-human IgG (Fc-specific)(BioCAN Scientific, Mississauga, ON), Texas Red-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA) and goat anti-rabbit IgG coupled to 5nm colloidal gold (Amersham, Oakville, ON).

4.2.3 Purification of immune and non-immune IgG

Immune and non-immune IgG were obtained by purifying IgG from the serum of a previously transfused patient with Glanzmann's thrombasthenia containing anti-GPIIb/IIIa antibodies, or the pooled sera of healthy donors (n=20). IgG was purified by caprylic acid precipitation and ammonium sulphate fractionation (Page and Thorpe, 1998). Isolated IgGs were dialyzed overnight in PBS (pH 7.4) and their protein concentrations were determined using the Bio-Rad protein assay (Richmond, CA). Purified IgG was analyzed on a 7% SDS-PAGE gel stained with Coomassie Blue, as described by Kelton *et al*, 1990. As a positive control, platelets were incubated with serum of a patient with post-transfusion purpura, containing anti-Pl^{A1} antibodies (specific antibodies to an epitope on GPIIb/IIIa).

4.2.4 Determination of reactivity of purified IgG

4.2.4.1 Radioimmunoprecipitation

Purified IgG was tested for reactivity against normal platelet surface proteins in a radioimmunoprecipitation experiment using radiolabeled normal platelet lysate as described by Kelton *et al*, 1990. Briefly, washed normal type O donor platelets were radiolabeled with ^{125}I-sodium

iodide using lactoperoxidase. The labeled platelets were solubilized with 1% Triton X-100 in Tris-buffered saline containing 10 mM EDTA, 0.1 mM PMSF and 0.02 mg/ml soybean trypsin inhibitor. Radiolabeled platelet lysate was incubated with 500 ul protein A-Sepharose CL-4B beads (Pharmacia Biotech, Baie d'Urfe, QC) and 100 ul (2 mg/ml) purified IgG. As a positive control, platelets were also incubated with serum from a patient with post-transfusion purpura, containing anti-Pl^{A1} antibodies. Following a two-hour incubation at room temperature, beads were washed 5 times and the bound complexes were eluted and analyzed using a 7.5-15% SDS-PAGE gel. All immunoprecipitations were run under reducing conditions using 5% 2-mercaptoethanol in the sample buffer.

4.2.4.2 Antigen capture assay

Purified IgG was also tested for reactivity against GPIIb/IIIa or GPIb/IX, in an antigen capture assay, according to Warner *et al* (1999). Briefly, whole blood from a normal type O donor was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1 mM theophylline and prostaglandin E_1 (1 ug/ml). Platelet-rich plasma (PRP) was obtained by centrifugation at 160g for 20 minutes at room temperature. PRP was incubated with purified IgG (400 ug/ml PRP) for 1 hour at room temperature. Positive controls were prepared by incubating PRP with serum from a patient with post-transfusion purpura (PTP), containing anti-Pl^{A1} antibodies. Platelets were then processed as described in section 2.2.3 and platelet lysate was analyzed in an antigen capture assay, as described in section 2.2.4.

4.2.5 Platelet incubations with purified immune and non-immune IgG

PRP or gel-filtered platelets resuspended in Tyrode's buffer (pH 7.4) from a normal type O donor were incubated with 300 ug of purified IgG (833 fg purified IgG added per platelet). Control platelets were incubated with buffer alone. Platelets were incubated for periods of 10 minutes, 3 hours or 20 hours at 37°C. Following incubation, platelets were washed and processed for confocal and electron microscopy studies. To determine whether platelets were activated by the addition of purified IgG, the platelet activation marker P-selectin was measured using flow cytometry as described in section 3.2.1.3.

4.2.6 Confocal microscopy studies

Platelets were fixed and permeabilized as described in section 3.2.1.4. Immunofluorescent labeling of platelets and laser scanning confocal microscopy were performed as described in sections 3.2.1.6 and 3.2.1.7.

4.2.7 Electron microscopy studies

Platelet samples were fixed and prepared for electron microscopy as outlined in section 3.4.1.2. Immuno-gold labeling and electron microscopy were performed as described in section 3.4.1.4. Gold particle enumeration, platelet organelle enumeration, and statistical analyses were performed as described in sections 3.4.15, 3.4.1.6 and 3.4.1.7.

4.3 Results

Immune and non-immune IgG were obtained by purifying IgG from the serum of a previously transfused patient with Glanzmann's thrombasthenia, containing anti-GPIIb/IIIa antibodies, or the pooled sera of normal donors (n=20), respectively. Purified IgG isolated from patient or normal control sera was analyzed on a 7% reduced SDS-PAGE gel and stained with Coomassie Blue (protein stain) (Figure 27A). As a positive control, the serum of a patient with post-transfusion purpura containing anti-Pl^{A1} antibodies (antibodies to an epitope on GPIIb/IIIa) was also included (lane 2). Purified non-immune IgG (lane 1) and purified immune IgG (lane 3) demonstrated bands which corresponded to albumin (~66 kDa) (major constituent of the sample buffer) and the heavy chain of the IgG molecule (~55 kDa). The light chain of the IgG molecule (~25 kDa) was observed in each lane at the gel bottom (not shown). Radioimmunoprecipitation of platelet proteins (Figure 27B) with purified non-immune IgG demonstrated an absence of reactivity (lane 1). In comparison, positive control IgG (lane 2) and purified immune IgG (lane 3), recognized proteins which corresponded to the GPIIb/IIIa complex (116 and 105 kDa).

When purified IgG was incubated with normal platelets and analyzed in an antigen capture assay, purified non-immune IgG demonstrated an absence of reactivity against GPIIb/IIIa or GPIb/IX. In the same assay, purified immune IgG and positive control IgG demonstrated reactivity against GPIIb/IIIa.

Using confocal microscopy, patterns of IgG distribution in fixed permeabilized and non-permeabilized normal platelets demonstrated a granular pattern of IgG staining within platelets and an absence of IgG staining on the surface of platelets (Figure 28). This pattern of IgG staining was

not changed after incubating normal platelets with non-immune IgG (Figure 28). In comparison, platelets incubated with purified immune IgG, demonstrated IgG staining both within, and on the surface of platelets (Figure 28). Staining of immune IgG on non-permeabilized platelets formed a rim pattern, similar to that described earlier for membrane glycoproteins, IIb/IIIa and Ib/IX (described in section 3.2.2). Measurement of the platelet activation marker P-selectin demonstrated that surface PAIgG in platelets incubated with immune IgG was not a result of platelet activation (3 hour incubation period) (Table 6). In platelets incubated with immune and non-immune IgG, the described PAIgG staining patterns were consistent over time (incubation periods of 10 minutes, 3 hours or 20 hours).

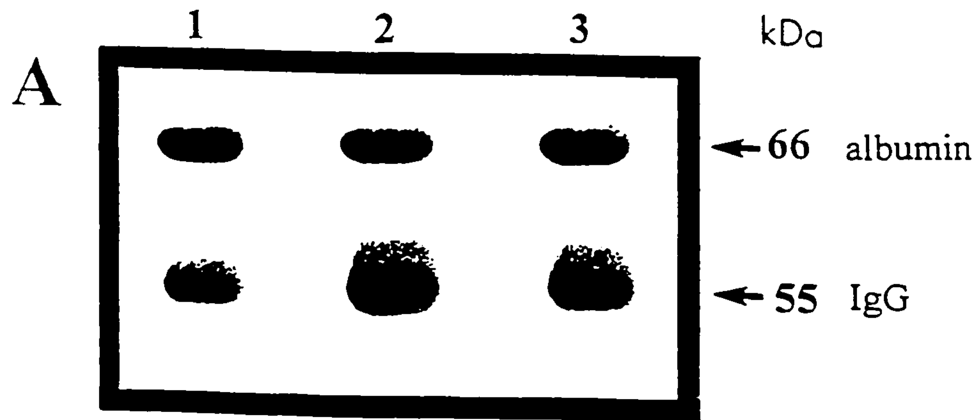
Immunogold labeling of IgG in normal resting platelets, was observed primarily within α-granules with little to no staining of the platelet surface membrane or channels of the open-cannalicular system (Figure 29A, B). After these platelets were incubated with immune IgG they demonstrated gold labeling within α-granules but also within channels of the open-cannalicular system and to a lesser extent on the platelet surface membrane (Figure 29E, F). Whereas, when the same platelets were incubated with non-immune IgG they demonstrated gold labeling primarily within α-granules with little to no staining of the platelet surface membrane or channels of the open-cannalicular system (Figure 29C, D).

Enumeration of gold particles (Tables 7-9) demonstrated that platelets incubated with immune IgG had significantly more gold particles within channels of the open-cannalicular system and on the platelet surface membrane in comparison to control platelets ($p<1.5x10^{-8}$). These observations were consistent whether platelets were incubated for 10 minutes (Table 7), 3 hours (Table 8), or 20 hours

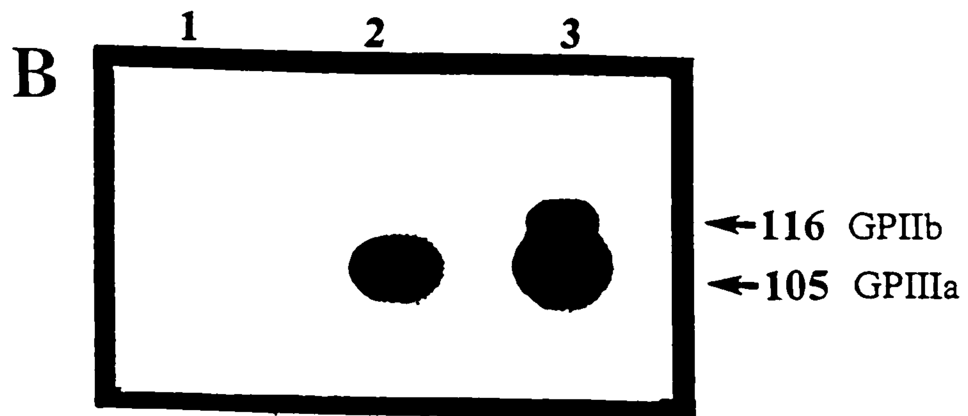
(Table 9) with immune IgG. In these platelets, immunogold labeling for IgG within α-granules was not significantly increased following incubation with immune IgG for 10 minutes or 3 hours, but was significantly increased following a 20 hour incubation period. Increased numbers of gold particles were not a reflection of an increase in average platelet area or increased numbers of α-granules per platelet (Tables 7-9). In comparison, enumeration of gold particles in platelets incubated with non-immune IgG demonstrated no significant difference in amount of gold particles in α-granules, channels of the open-cannalicular system or on the platelet surface membrane from that of normal controls. Additionally, an increase in gold labeling within platelets was not observed over time (Tables 7-9).

Figure 27 Analysis of purified IgG using gel-electrophoresis and radioimmunoprecipitation.

Purified IgG isolated from a pool of normal sera (n=20) (lane 1) and the serum of a previously transfused patient with Glanzmann's thrombasthenia (lane 3) were analyzed on a 7% reduced SDS-PAGE gel (A). As a positive control, serum of a patient with post-transfusion purpura, containing anti-Pl^{A1} antibody, was included (lane 2). Bands which corresponded to albumin (~66 kDa) (major constituent of the sample buffer) and the heavy chain of the IgG molecule (~55 kDa) were observed. The light chain of the IgG molecule (~25 kDa), was also observed in each lane, at the gel bottom (not shown). Radioimmunoprecipitation of platelet proteins (B) with purified non-immune IgG demonstrated an absence of reactivity (lane 1). In comparison, purified immune IgG (lane 3) recognized proteins which corresponded to the GPIIb/IIIa complex (116 and 105 kDa).



A
1
2
3
kDa
66 albumin
55 IgG



B
1
2
3
116 GPIIb
105 GPIIIa

Figure 28 Immunolocalization of PAIgG following incubation of normal resting platelets *in vitro* with purified IgG, using laser scanning confocal microscopy.

Platelets were incubated with rabbit anti-human IgG and Texas-Red-conjugated goat anti-rabbit IgG antibody. Patterns of IgG distribution in fixed permeabilized and non-permeabilized control platelets, using laser scanning confocal microscopy demonstrated a granular pattern of IgG staining within platelets (permeabilized), and an absence of IgG staining on the surface of platelets (non-permeabilized) (A). An identical pattern of IgG staining was observed for platelets incubated with non-immune IgG (B). In comparison, platelets incubated with purified immune IgG demonstrated IgG staining both within (permeabilized) and on the surface of platelets (non-permeabilized) (C). Staining of immune IgG on non-permeabilized platelets formed a rim pattern similar to that observed for membrane glycoproteins IIb/IIIa and Ib/IX (not shown). In both platelets incubated with immune and non-immune IgG, the described PAIgG staining patterns were consistent over time (incubation periods of 10 minutes, 3 hours or 20 hours).

The Localization of IgG in Type O Normal Donor Platelets following a 3 Hour Incubation with Nonimmune or Immune IgG, using LSCM

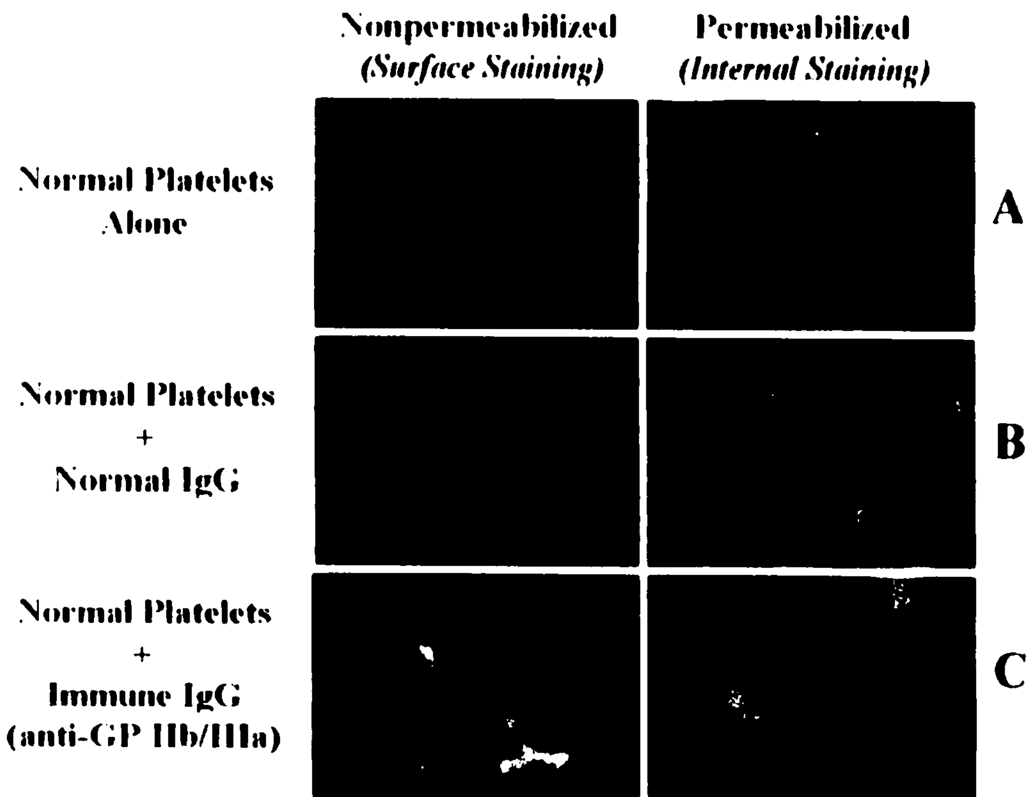


TABLE 6

Measurement of Platelet Activation following incubation of Normal Platelets with Purified Immune or Nonimmune IgG

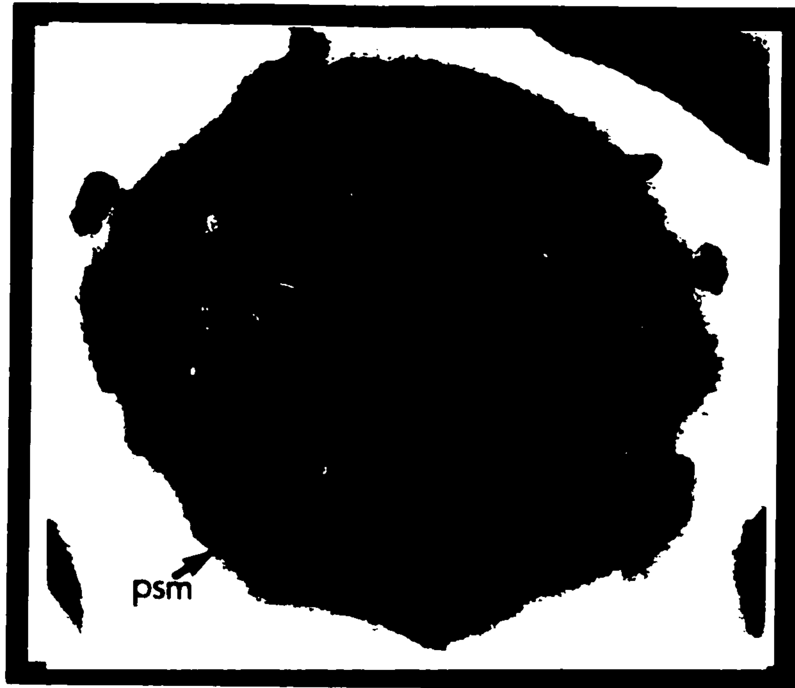
INCUBATIONS	NONACTIVATED
	% of Platelets expressing P-Selectin
Platelets + Buffer	2.9
Platelets + Immune IgG	3.3
Piatelets + Nonimmune IgG	4.8

Figure 29 Immunogold labeling of PAIgG in normal resting platelets following incubation with purified immune or non-immune IgG.

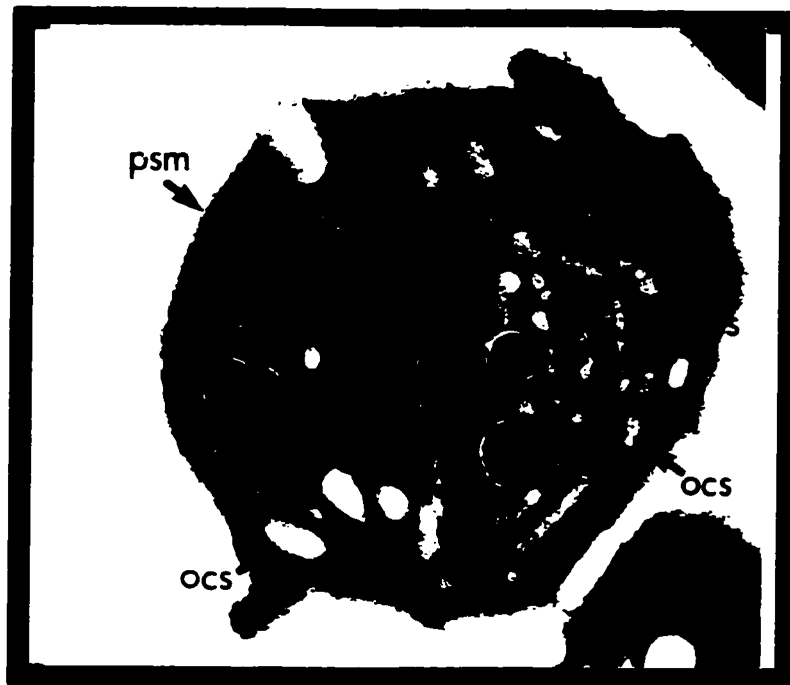
Immunogold labeling of IgG in normal resting platelets was observed primarily within α-granules with little to no staining of the platelet surface membrane or channels of the open-cannalicular system (A, B). Similarly, after these platelets were incubated with non-immune IgG gold labeling was observed primarily within α-granules with little to no staining of the platelet surface membrane or channels of the open-cannalicular system (C, D). Whereas, after these same platelets were incubated with immune IgG, gold labeling was observed within α-granules, channels of the open-cannalicular system and to a lesser extent on the platelet surface membrane (E, F).

Original Magnification: X 35, 000 (A, B, C); X 44, 000 (D, E); X 36, 000 (F).

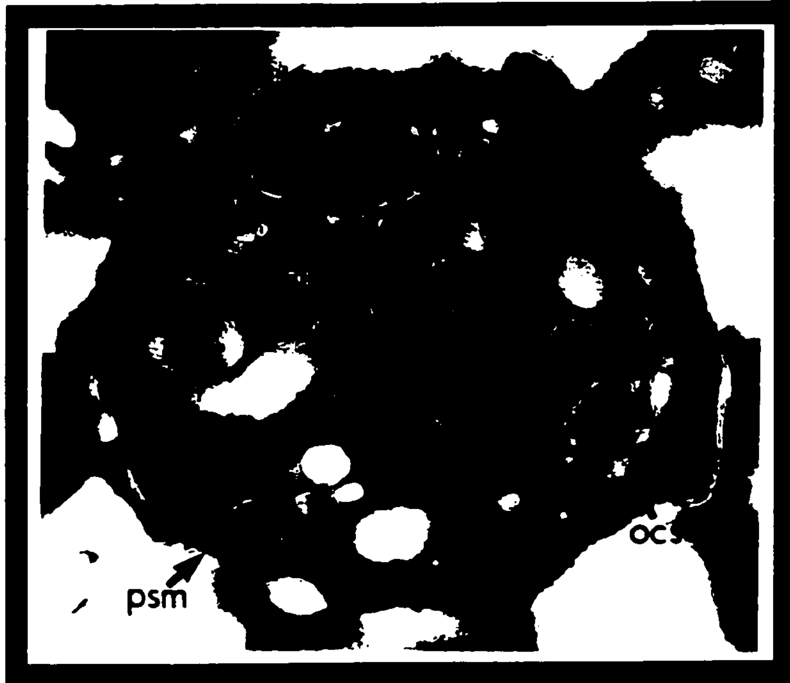
A



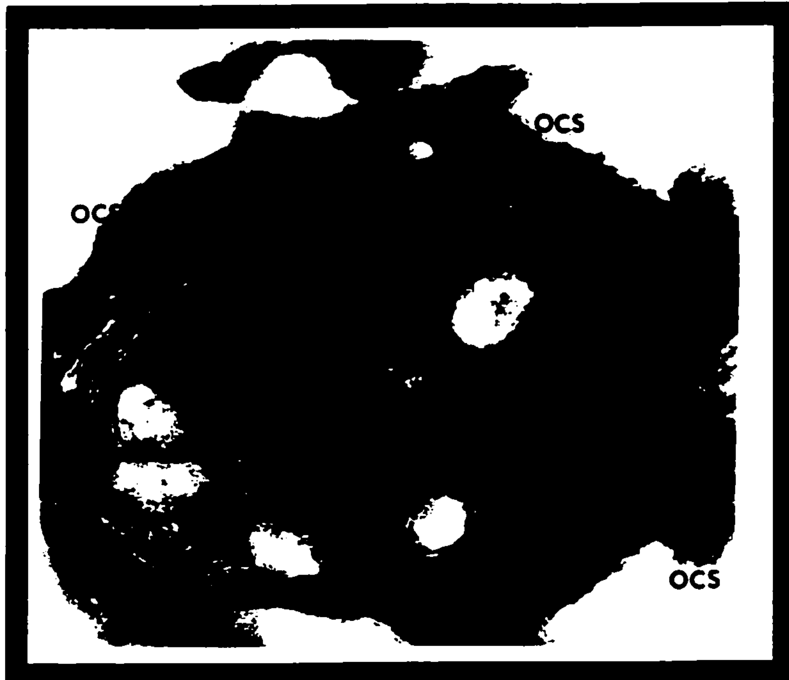
B



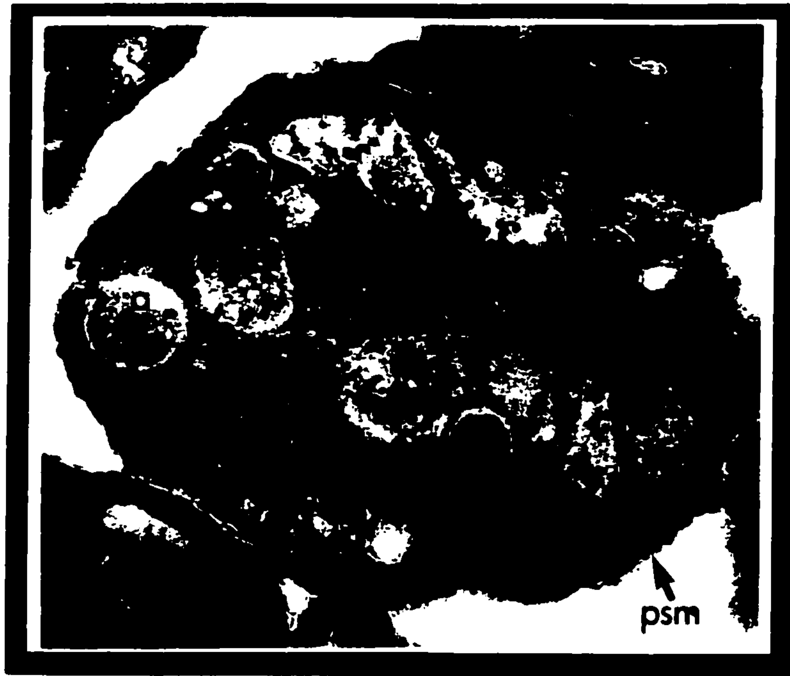
C



D



E



F

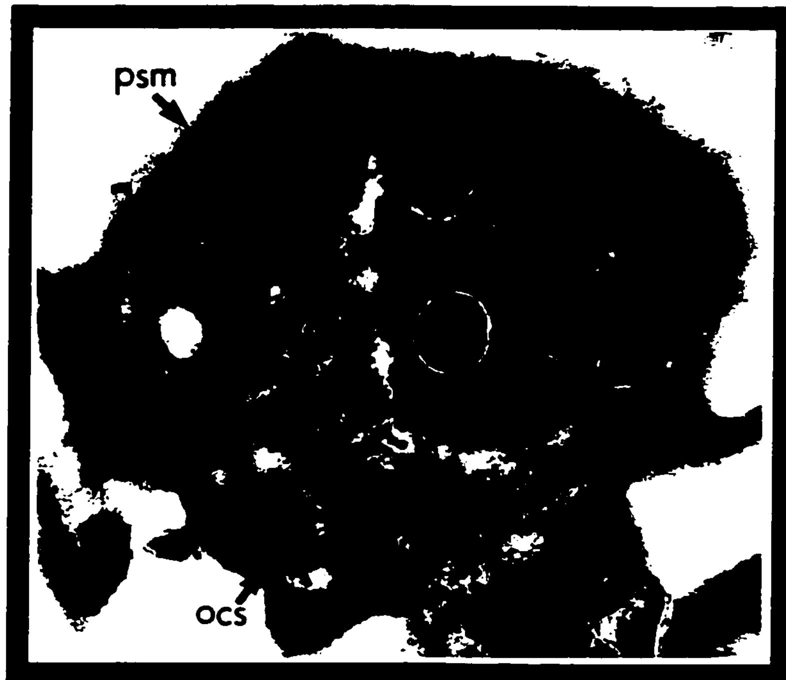


TABLE 7

Average Number of Counted Gold Particles per Platelet Section following Incubation with Purified Immune or Nonimmune IgG for 10 minutes at 37°C.

	PLATELETS ALONE	PLATELETS PLUS NORMAL IgG	PLATELETS PLUS IMMUNE IgG
Number of Counted Platelets	65	65	65
Average Platelet Area (um^2)	2.85±1.35	2.73±1.25	2.91±1.17
# Alpha Granules/Platelet	9.24±5.96	10.41±7.94	9.51±6.14
# Mitochondria/Platelet	2.95±2.23	3.71±2.59	3.62±2.22
TOTAL (Alpha + Mitochondria)	12.20±7.19	14.16±9.49	13.15±7.15
# Gold - Surface/Platelet	0.06±0.29	0.23±0.62	8.19±2.86
# Gold - OCS/Platelet	0.09±0.38	0.28±0.29	7.77±5.52
# Gold - Alpha/Platelet	12.34±10.42	11.67±12.95	13.04±11.94
# Gold - Other/Platelet	2.74±3.01	3.48±3.89	7.67±5.52
TOTAL # GOLD	15.21±11.42	15.63±15.34	31.67±17.98
# Gold/um^2	5.23±2.65	5.21±2.82	10.67±8.29

Data are reported as means ± standard deviation (SD). Shaded boxes indicate those values which were significantly higher than values obtained from control platelets (platelet alone with no exogenous IgG added); $p<8.2044\times10^{-10}$.

TABLE 8

Average Number of Counted Gold Particles per Platelet Section following Incubation with Purified Immune or Nonimmune IgG for 3 hours at 37°C.

	PLATELETS ALONE	PLATELETS PLUS NORMAL IgG	PLATELETS PLUS IMMUNE IgG
Number of Counted Platelets	62	62	62
Average Platelet Area (um^2)	2.81±0.89	3.27±1.32	2.76±1.20
# Alpha Granules/Platelet	10.87±5.17	8.52±5.06	10.26±4.82
# Mitochondria/Platelet	4.12±2.49	2.85±2.14	3.26±2.03
TOTAL (Alpha + Mitochondria)	14.98±6.57	11.39±6.37	13.52±6.12
# Gold - Surface/Platelet	0.22±0.73	0.07±0.32	6.04±3.86
# Gold - OCS/Platelet	0.17±0.56	0.19±0.69	6.52±4.02
# Gold - Alpha/Platelet	12.12±7.51	10.36±6.86	13.97±8.27
# Gold - Other/Platelet	2.80±2.85	3.63±3.85	13.89±9.75
TOTAL # GOLD	15.32±9.12	14.19±9.00	40.43±17.97
# Gold/um^2	5.63±2.98	4.36±1.97	15.06±4.66

Data are reported as means ± standard deviation (SD). Shaded boxes indicate those values which were significantly higher than values obtained from control platelets (platelet alone with no exogenous IgG added); $p<3.457 \times 10^{-12}$.

TABLE 9

Average Number of Counted Gold Particles per Platelet Section following Incubation with Purified Immune or Nonimmune IgG for 20 hours at 37°C.

	PLATELETS ALONE	PLATELETS PLUS NORMAL IgG	PLATELETS PLUS IMMUNE IgG
Number of Counted Platelets	60	60	60
Average Platelet Area (um^2)	2.95±1.13	2.78±0.97	2.96±1.05
# Alpha Granules/Platelet	10.14±4.32	9.47±4.1	9.66±4.58
# Mitochondria/Platelet	3.87±2.16	3.59±2.25	3.08±2.19
TOTAL (Alpha + Mitochondria)	14.05±6.67	13.1±6.13	12.79±5.9
# Gold - Surface/Platelet	0.34±0.11	0.25±0.32	**12.19±3.42**
# Gold - OCS/Platelet	0.38±0.09	0.41±0.28	**8.95±2.67**
# Gold - Alpha/Platelet	13.04±4.19	12.57±5.37	**24.65±8.90**
# Gold - Other/Platelet	2.78±1.96	2.85±1.48	**8.12±5.31**
TOTAL # GOLD	16.43±7.33	15.98±6.51	**53.87±18.98**
# Gold/um^2	5.56±1.87	5.71±1.97	**18.26±5.83**

Data are reported as means ± standard deviation (SD). Shaded boxes indicate those values which were significantly higher than values obtained from control platelets (platelet alone with no exogenous IgG added); $p<3.584x10^{-8}$.

4.4 Summary

In these studies, possible pathways of IgG internalization were investigated, by determining the immunolocalization of IgG in normal platelets following incubation with anti-platelet antibody or non-immune IgG. Using this approach, it was possible to quantitate IgG within various platelet organelles, over time, and compare patterns of IgG distribution in platelets incubated with immune versus non-immune IgG. In these studies, immune IgG was isolated from the serum of a previously transfused patient with Glanzmann's thrombasthenia, containing anti-GPIIb/IIIa antibodies. Analysis of purified IgG demonstrated that immune IgG recognized GPIIb/IIIa and demonstrated reactivity against this complex in an antigen capture assay. Non-immune IgG was isolated from a pool of normal sera, collected from healthy controls (n=20) and demonstrated no reactivity against normal platelet proteins. The addition of both purified immune or non-immune IgG to normal platelets did not result in platelet activation (following 3 hour incubation).

Immunofluorescent labeling of IgG in platelets incubated with non-immune IgG demonstrated an IgG granular pattern of staining within platelets and an absence of IgG staining on the surface of platelets. These results were similar to that observed for normal control platelets. In comparison, IgG immunofluorescence in platelets incubated with immune IgG demonstrated an IgG granular pattern of staining within platelets, and rim pattern staining on the surface of platelets. This rim pattern staining was similar to that observed in non-permeabilized platelets from patients with ITP and in normal control platelets stained for GPIIb/IIIa or GPIb/IX. For platelets incubated with both immune and non-immune IgG, the described IgG staining patterns were consistent over time (10-minute, 3-hour and 20-hour incubation periods).

Immunogold labeling for IgG in platelets incubated with non-immune IgG demonstrated localization of gold particles primarily within α-granules with little to no staining of the platelet surface membrane or channels of the open-cannalicular system. This was similar to that observed for control platelets. Enumeration of gold particles demonstrated that platelets incubated with non-immune IgG had similar amounts of gold particle labeling (in α-granules, channels of the open-cannalicular system, and on the platelet surface membrane) as control platelets with no increase in gold labeling, over time. In comparison, platelets incubated with immune IgG, demonstrated gold labeling primarily within α-granules and channels of the open-cannalicular system. Labeling of the platelet surface membrane was also observed, albeit to a much less extent. Enumeration of gold particles, demonstrated that platelets incubated with immune IgG had significantly more gold particles within channels of the open-cannalicular system and on the platelet surface membrane in comparison to control platelets or platelets incubated with non-immune IgG ($p<1.5 \times 10^{-8}$). These observations were consistent whether platelets were incubated for 10 minutes, 3 hours, or 20 hours. Immunogold labeling within α-granules was not significantly increased in platelets incubated with immune IgG for short periods of time (10 minutes or 3 hours) but demonstrated a significant increase following a longer incubation time (20 hours). In these platelets, increased numbers of gold particles were not a reflection of an increase in average platelet area or increased numbers of α-granules per platelet.

These results suggest that platelet internalization of immune versus non-immune IgG may differ. Pathways of immune IgG internalization may involve binding to the platelet surface membrane and transport via channels of the open-cannalicular system to α-granules over time. In comparison, pathways of non-immune IgG internalization may occur primarily at a megakaryocyte level with little to no internalization at a circulating platelet level.

CHAPTER 5

The immunolocalization of PAIgG on the platelet surface over time.

5.1 Introduction

The origin of IgG on the surface of platelets of patients with non-immune thrombocytopenia is not known. Additionally, it is not clear why these IgG-sensitized platelets escape clearance by phagocytic cells of the reticuloendothelial system. One hypothesis suggests that elevated surface PAIgG in patients with non-immune thrombocytopenia results from IgG leakage from α-granules onto the surface of platelets incubated *in vitro* over time. In this way, surface PAIgG may represent non-specific α-granule IgG and is pathologically unimportant. In these studies, leakage of IgG from platelets incubated *in vitro*, over time, was investigated. The purpose of these studies was to determine the immunomorphologic characteristics of surface PAIgG in platelets following; (1) immediate processing, or (2) after an overnight incubation period. In this way, IgG leakage onto the surface of platelets was investigated by determining patterns of surface PAIgG distribution, over time.

5.2 Materials and methods

5.2.1 Patients and controls

In this investigation, 3 adult patients with ITP, 9 adult patients with non-immune hematological disorders (thrombocytopenic, n=3; normal platelet count, n=6) and 5 healthy adult controls were studied. Patients categorized with non-immune thrombocytopenia, included two patients recovering from bone marrow transplants and one patient with thyroid cancer. None of these patients had received platelet or whole blood transfusions at the time of study. The average platelet count for

patients with non-immune thrombocytopenia was 63×10^9/L and average total PAIgG was 12fg/platelet (normal PAIgG value is <5fg/platelet). The average platelet count for patients with ITP was 50×10^9/L and average total PAIgG was 18fg/plt. The average platelet count for patients with non-immune hematological disorders and a normal platelet count was 345×10^9/L and average total PAIgG was <3fg/plt. In an antigen capture assay (outlined in section 2.2.4) platelet lysates from all patients with ITP demonstrated detectable anti-GPIIb/IIIa antibody, whereas all non-immune patients and normal controls tested negative.

5.2.2 Platelet collection and preparation

Whole blood was collected into acid citrate dextrose (ACD, pH 4.5, 6:1, vol:vol) containing 1mM theophylline and prostaglandin E_1 (1 ug/ml) and centrifuged at 120 g for 20 minutes to obtain platelet-rich plasma. As a positive control, normal PRP was incubated (30 minutes at room temperature) with serum from a patient with post-transfusion purpura (PTP) containing anti-Pl^{A1} antibodies. Platelets were isolated by gel filtration as described in section 3.2.1.2 and either; (1) fixed immediately or (2) fixed following an overnight incubation at room temperature. Platelet fixation was performed as described in section 3.2.1.4.

5.2.3 Antibodies

Antibodies used in flow cytometry studies included FITC-conjugated monoclonal anti-human IgG (CAG-2), as described by Horsewood *et al* (1991) and FITC-conjugated anti-P-selectin monoclonal antibody (Cedarlane Laboratories, Guelph, ON). Antibodies used for immunofluorescent labeling studies included rabbit anti-human IgG (Fc-specific) (BioCAN Scientific, Mississauga, ON) and

Texas Red (TR)-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA).

5.2.4 Flow cytometry

P-selectin (activation marker) and PAIgG, were measured on non-permeabilized platelets using flow cytometry as described in sections 3.2.1.3, 3.3.1.4 and 3.3.1.5.

5.2.5 Laser scanning confocal microscopy

PAIgG on non-permeabilized platelets was examined using confocal microscopy as described in sections 3.2.1.6 and 3.2.1.7.

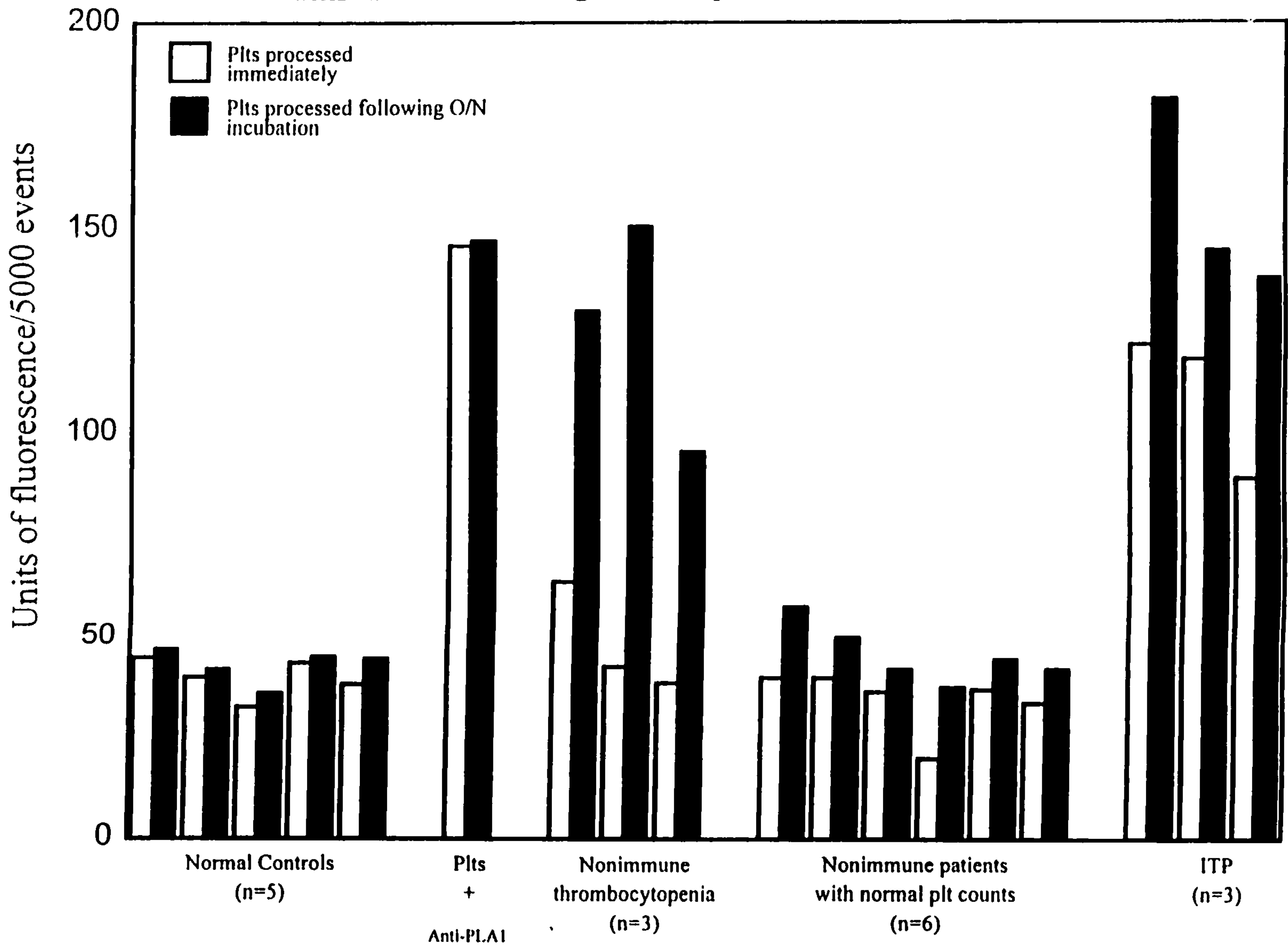
5.3 Results

In these studies, flow cytometry was used to provide a quantitative measure of surface PAIgG in fixed non-permeabilized platelets. In platelets from patients with non-immune hematological disorders (with normal platelet counts) (n=6) and normal controls (n=5), surface PAIgG values increased slightly following an overnight incubation *in vitro*(Figure 30, Table 10). Positive control platelets (incubated with anti-Pl^{A1} antibodies) demonstrated a similar slight increase. In these patient and control platelets, increased expression of a platelet activation marker (P-selectin) was also observed following overnight incubation (Table 10). Corresponding confocal microscopy images of these platelets demonstrated an absence of surface PAIgG on patient and control platelets which were either fixed immediately or fixed following an overnight incubation (Figure 31).

In platelets from patients with ITP (n=3) and non-immune thrombocytopenia (n=3), surface PAIgG and P-selectin expression, increased following overnight incubation (Figure 30, Table 10). In these patients, the increase in surface PAIgG was dramatic. Corresponding confocal microscopy images of patient platelets demonstrated rim pattern staining in ITP platelets for both platelets fixed immediately and platelets fixed following an overnight incubation (Figure 31). In comparison, platelets from patients with non-immune thrombocytopenia, demonstrated different patterns of surface PAIgG depending on the length of time in which platelets were allowed to incubate, prior to fixation. Platelets which were fixed immediately demonstrated an absence of surface PAIgG, whereas platelets which were incubated overnight demonstrated IgG staining on the platelet surface (Figure 31). In these platelets, IgG immunofluorescence appeared as several distinct focal points around the edge of the platelet forming a "beaded necklace"appearance. This pattern of IgG staining on the platelet surface was different from the rim pattern observed in ITP patients but was similar to a pattern of IgG staining observed in platelets from a group of transfused patients with non-immune thrombocytopenia (Figure 32). In this small group of patients (n=5), patients had received transfusions of donor whole blood prior to platelet collection. Additionally, collected whole blood samples were unavoidably incubated overnight prior to processing. Because each patient had received a recent blood transfusion it was not possible to determine whether the analyzed platelets were of patient origin or transfused donor origin. For this reason, cautious interpretation of IgG staining on the surface of these platelets is required.

Figure 30 Quantitative flow cytometric measurement of surface PAIgG in non-permeabilized platelets processed immediately, or following an overnight incubation.

PAIgG was measured using flow cytometry and expressed as units of fluorescence. Surface PAIgG measurements on non-permeabilized platelets from normal controls (n=5), patients with non-immune hematological disorders (thrombocytopenic, n=3; normal platelet count, n=6), and ITP patients (n=3) demonstrated an increase in surface PAIgG following an overnight incubation. This increase was very slight in controls and non-immune patients with normal platelet counts but dramatic in patients with ITP and non-immune thrombocytopenia. Positive control platelets (platelets incubated with anti-Pl^{A1} antibody) demonstrated a slight increase in surface PAIgG.



Measurement of PAIgG on nonpermeabilized fixed platelets.
Plts processed immediately
Plts processed following O/N incubation
Units of fluorescence/5000 events
200
150
100
50
0
Normal Controls
(n=5)
Plts
+
Anti-PLA1
Nonimmune
thrombocytopenia
(n=3)
Nonimmune patients
with normal plt counts
(n=6)
ITP
(n=3)

TABLE 10 - Measurement of Platelet Surface IgG and Platelet Activation in Non-permeabilized Platelets processed immediately, or following O/N Incubation

	PAIgG		P-Selectin	
	Processed Immediately	O/N Incubation	Processed Immediately	O/N Incubation
Normal Controls (n=5)	44.9	47	6.2	7.9
	40.1	42	10.8	16.2
	32.8	36.1	10.4	17
	43.7	45.1	9.3	12.7
	38.3	44.5	11.1	15.8
Normal Control plus anti-PLA1	146	147.2	10.4	17.1
Nonimmune Thrombocytopenia (n=3)	64	130	14.2	29
	42.6	150.7	9.5	9.25
	38.6	95.4	9.2	12.4
Nonimmune (Normal Plt. count) (n=6)	40	58	10	32
	40	50	10	24.1
	36.5	42	7.2	9.5
	20.2	37.5	8.5	9
	36.9	44.4	8.2	16.8
	33.6	42	10.2	15.3
ITP (n=3)	121.7	181.1	14.2	15.1
	118.3	145	10.3	19.6
	89	138.5	9.5	18

Figure 31 Immunolocalization of surface PAIgG in non-permeabilized platelets processed immediately, or following an overnight incubation.

Platelets were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Analysis of IgG immunofluorescence in platelets from normal controls (A) and non-immune patients with normal platelet counts (B) demonstrated an absence of IgG on the surface of platelets whether processed immediately, or processed following an overnight incubation. Platelets from patients with non-immune thrombocytopenia (C_1, C_2, C_3), which were processed immediately demonstrated an absence of IgG immunofluorescence on the platelet surface. In comparison, platelets processed following an overnight incubation demonstrated IgG staining on the surface of platelets. This pattern of IgG immunofluorescence appeared as several distinct focal points around the edge of the platelet forming a "beaded necklace"appearance. This pattern was different from the rim pattern of IgG staining observed on ITP platelets (D) which were either processed immediately or following an overnight incubation.

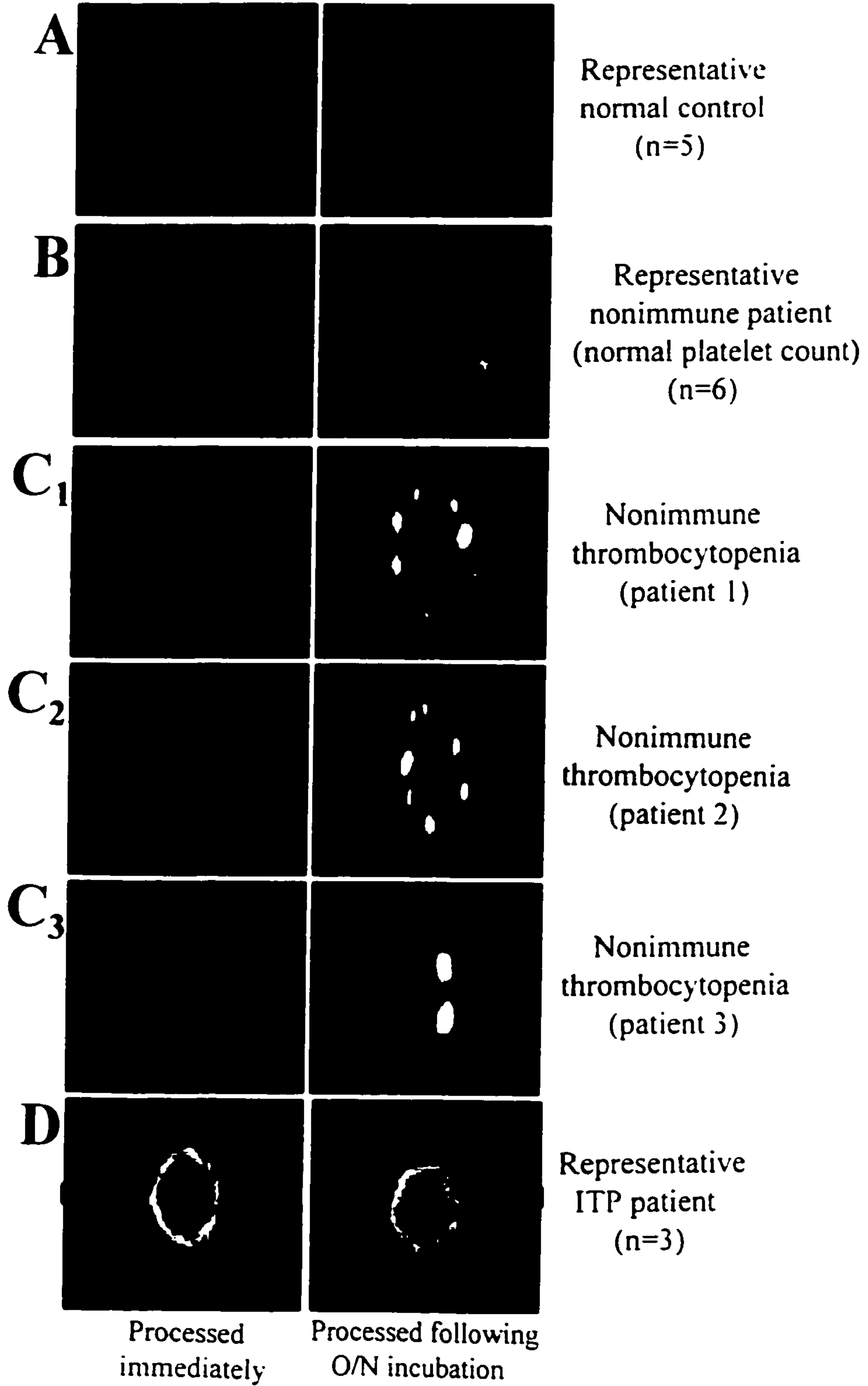
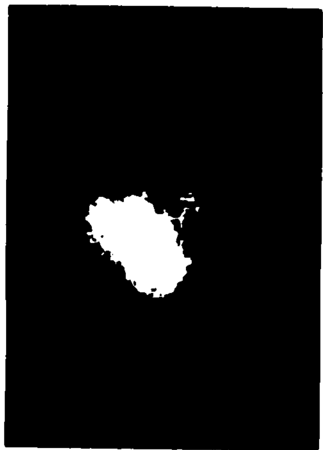

NONPERMEABILIZED PLATELETS
A
Representative normal control (n=5)
B
Representative nonimmune patient (normal platelet count) (n=6)
C1
Nonimmune thrombocytopenia (patient 1)
C2
Nonimmune thrombocytopenia (patient 2)
C3
Nonimmune thrombocytopenia (patient 3)
D
Representative ITP patient (n=3)
Processed immediately
Processed following O/N incubation

Figure 32 Immunolocalization of surface PAIgG in non-permeabilized platelets from transfused thrombocytopenic patients, following delayed platelet processing.

Platelets from transfused patients with non-immune thrombocytopenia (n=5) which incubated overnight prior to processing were incubated with rabbit anti-human IgG and Texas-Red conjugated goat anti-rabbit IgG antibody. Analysis of IgG immunofluorescence in non-permeabilized fixed platelets demonstrated several distinct focal points around the edge of the platelet forming a "beaded necklace" appearance. Because these patients had received whole blood transfusions prior to platelet collection it was not possible to determine whether the analyzed platelets were of patient origin or transfused donor origin. For this reason, cautious interpretation of IgG staining on the surface of these platelets is required.

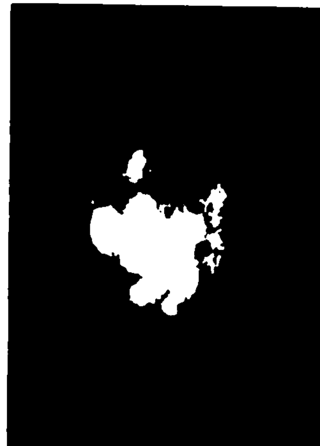
Permeabilized Platelets


Transfused Patient 1

Transfused Patient 2

Transfused Patient 3

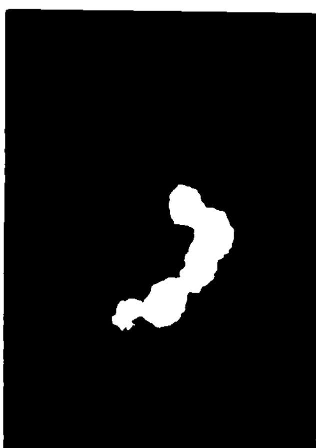
Transfused Patient 4


Transfused Patient 5

Nonpermeabilized Platelets

Transfused Patient 1

Transfused Patient 2


Transfused Patient 3

Transfused Patient 4

Transfused Patient 5

5.4 Summary

In these studies, leakage of IgG from platelets incubated *in vitro* over time was investigated. The purpose of these studies was to determine the immunomorphologic characteristics of surface PAIgG in platelets following; (1) immediate processing, or (2) after an overnight incubation period. The results of these studies demonstrated that in non-immune patients with normal platelet counts and healthy controls there is an absence of detectable IgG immunofluorescence on the surface of non-permeabilized platelets. This observation is consistent whether platelets are processed immediately, or following a delay in processing (following an overnight incubation). In platelets from patients with ITP, IgG is detected on the surface of platelets, forming a rim pattern. This pattern of IgG staining on the surface of platelets was consistent whether platelets were processed immediately or following a delay in processing.

In comparison, platelets from patients with non-immune thrombocytopenia consistently demonstrate an absence of IgG staining on the platelet surface when there is no delay in platelet processing. However, when these same platelets are allowed to sit overnight prior to processing previously unobserved IgG staining is seen on the platelet surface. In these platelets focal points of IgG were observed around the platelet edge forming a "beaded necklace"appearance. This type of pattern differed from that observed in ITP patients but was similar to that observed for transfused patients in which PRP samples sat overnight prior to processing. However, in this small group of transfused patients it was not possible to determine whether the analyzed platelets were of patient origin or transfused donor origin. For this reason, cautious interpretation of IgG staining on the surface of these platelets is required.

These results showed that the immunomorphologic characteristics of surface PAIgG in platelets from patients with non-immune thrombocytopenia change over time. Measurement of a platelet activation marker demonstrated that patient platelets were not activated to a greater degree during overnight incubation than control platelets. The time-dependent appearance of IgG on the surface of these platelets may reflect the elevated amount of PAIgG within these platelets since non-immune patients with normal PAIgG values show an absence of surface PAIgG. Additionally, these results demonstrate different patterns of surface PAIgG in patients with immune versus non-immune thrombocytopenia. It is suggested that *in vitro* leakage of α-granule IgG onto the surface of platelets may account for the origin of some PAIgG in platelets of patients with non-immune thrombocytopenia.

CHAPTER 6

Discussion

6.1 Thesis objectives

For many years, assays for platelet-associated IgG (PAIgG) were routinely used as an adjunct in the diagnosis of ITP. However, elevated levels of PAIgG are not specific for ITP and are also observed in thrombocytopenic disorders not thought to be immune-mediated. Despite agreement that much of the surface PAIgG in patients with ITP represents anti-platelet autoantibody, the origin of IgG on the surface of platelets of patients with non-immune thrombocytopenia is not known. Additionally, it is not clear why these IgG-sensitized platelets escape destruction by phagocytic cells of the reticuloendothelial system. The objectives of this thesis were to investigate possible biological explanations for the elevated PAIgG in adult patients with ITP and non-immune thrombocytopenia. Using ultrastructural techniques the immunomorphologic characteristics of PAIgG from patients with ITP and non-immune thrombocytopenia were determined. The results of these investigations provided further insight into mechanisms of IgG accumulation in platelets and suggested a novel explanation for why IgG-sensitized platelets in patients with non-immune thrombocytopenia are not prematurely destroyed.

6.2 Biological interpretation of elevated PAIgG in platelets from patients with ITP and non-immune thrombocytopenia - Examination of the most currently accepted hypothesis

Several hypotheses have been proposed to account for the observed elevations of PAIgG in patients with immune and non-immune thrombocytopenia. These hypotheses vary from proposed physical alterations of the platelet ultrastructure to proposed functional alterations that promote platelet

activation or endocytosis. Whereas some of these hypotheses account for elevated total PAIgG, others account for elevated surface PAIgG. Currently, there is no hypothesis which provides an adequate biological explanation for the increased amounts of PAIgG on both the platelet surface, and within the platelet, of patients with immune and non-immune thrombocytopenia.

The most currently accepted hypothesis suggests that elevated PAIgG in ITP and non-immune thrombocytopenia may be analogous to accelerated erythropoiesis in immune or non-immune hemolytic anemia (George, 1991). In this hypothesis it is postulated that elevated PAIgG in ITP and non-immune thrombocytopenia is a measure of the production of large, young platelets reflecting the degree of increased marrow stimulation (George, 1991). As a result of the increased platelet size, α-granule content is increased with increased amounts of IgG and other α-granule proteins. This hypothesis is supported by two key observations. Firstly, serologic assays have indicated that total PAIgG in patients with ITP correlates with measures of total platelet protein, platelet volume and platelet density (Levin and Bessman, 1983; Pfueller *et al*, 1986). Secondly, flow cytometry studies have indicated that the largest platelets in ITP contain the most IgG (George, 1991). However, this hypothesis has been challenged by conflicting reports from other investigative groups. For example, Kelton *et al* (1979), Holme *et al* (1988) and Heaton *et al* (1988), found no relationship between increased levels of PAIgG and platelet size despite some patients having severe thrombocytopenia. Moreover, Illes *et al* (1987) showed no difference in platelet density between normal controls, ITP patients and patients with non-immune thrombocytopenic disorders.

In this thesis project, I investigated this hypothesis directly by comparing measurements of α-granule proteins and parameters of platelet size between normal controls and patients with ITP or non-immune thrombocytopenia. Collectively, the results of these investigations provided additional evidence that elevated PAIgG in thrombocytopenic patients is not a reflection of the production of larger platelets with increased α-granule content. Firstly, when two different subsets of α-granule proteins (endogenous and exogenous proteins) were measured in platelets from patients with ITP and non-immune thrombocytopenia increased amounts of plasma-derived (exogenous) α-granule platelet proteins (IgG, albumin and fibrinogen) with no parallel increase in megakaryocyte-synthesized (endogenous) α-granule proteins (β-TG and PF4) were observed. In these studies, increased amounts of exogenous proteins were not observed in all individual patients with ITP and non-immune thrombocytopenia. These findings suggest that there may be two subsets of thrombocytopenic patients; those with increased amounts and those with no increase. The reason for this disparity is unclear but may be related to the magnitude of a stimulus for plasma protein accumulation or may reflect varying degrees of thrombocytopenia. However, in these studies I focused on whether there was a difference in the overall mean value of measured α-granule proteins between different patient groups rather than individual patients. Hence, when assayed by the same technique, patients with ITP and patients with non-immune thrombocytopenia had significantly higher mean total exogenous protein values compared to normal controls. It is suggested that these differences are not a reflection of the platelet preparation or assay technique but rather a reflection of the patient population as a whole. These results suggest that in patients with ITP and non-immune thrombocytopenia the platelet stores of some but not all α-granule proteins are altered. Moreover, the restriction of quantitative abnormalities to the pool of exogenous α-granule proteins suggests that

there is an abnormality in the pathway of acquisition of plasma proteins, including IgG, in patients with ITP and non-immune thrombocytopenia.

Secondly, an overall increase in mean platelet volume was not observed in patients with ITP or non-immune thrombocytopenia compared to normal controls. Although some individual patients had increased mean platelet volumes these differences did not reach statistical significance for the patient group as a whole. Once again, I focused on whether there was a difference in the overall mean platelet volume between different patient groups rather than individual patients. These results indicate that platelets from patients with ITP and non-immune thrombocytopenia were not increased in size compared to normal controls.

Lastly, direct visualization of platelet sections from 6 well characterized patients with ITP or 6 well characterized patients with non-immune thrombocytopenia under an electron microscope, demonstrated no increase in average number of α-granules per platelet section or average platelet section area (um^2) compared to normal controls.

The results of these investigations differed from previous reports that platelets from thrombocytopenic patients are significantly increased in size and total α-granule content (Levin and Bessman, 1983; Pfueller *et al*, 1986; Zucker-Franklin, 1981). There may be several reasons for these differences. Firstly, the degree of thrombocytopenia in the patients studied may have differed. In this thesis project a minimum platelet count of $20x10^9/L$ was required for processing considerations. In this way, patients with more severe thrombocytopenia were not analyzed and evaluation of any large

platelets produced in these patients would have been excluded. However, irrespective of degree of thrombocytopenia, thrombocytopenic patients studied in this thesis project consistently demonstrated elevated PAIgG values which did not correspond to abnormalities of platelet size or numbers of α-granules per platelet. Secondly, previous investigations using small numbers of patients may have reflected individual patient heterogeneity rather than differences between patient groups as a whole. For example, previous reports of megathrombocytes in patients with ITP involved studies of a single patient with severe thrombocytopenia (Zucker-Franklin, 1981). In this thesis project I evaluated platelets from a large number of random ITP and non-immune patients and focused on whether there was a difference between different patient groups rather than individual patients. Using a number of different techniques the results of this thesis project consistently indicated that elevated levels of PAIgG observed among thrombocytopenic patients are not attributable to alterations in the physical characteristics of platelets including increased platelet size, increased numbers of α-granules per platelet or increased α-granule content but reflect an isolated quantitative abnormality in the pool of exogenous α-granule proteins.

Despite evidence that platelet stores of exogenous α-granule proteins are elevated in patients with ITP and non-immune thrombocytopenia the biological significance of these elevations is not known. In von Willebrand disease pathological elevations in platelet fibrinogen have been reported in some patients (McKeown *et al*, 1993). However these elevations have not been reported by other investigative groups (Chen *et al*, 1998).The pathological significance of elevated plasma proteins in thrombocytopenic patients is not known. It has been hypothesized that some accumulated plasma proteins such as IgG, fibrinogen and albumin may enhance platelet aggregation or serve to bind

toxins and concentrate them within platelets (Koch-Weser and Sellers, 1976). Alternatively, elevations of plasma proteins may be functionally unimportant occurring as a non-specific result of enhanced fluid-phase endocytosis in megakaryocytes and/or platelets.

6.3 Biological interpretation of elevated PAIgG in platelets from patients with ITP and non-immune thrombocytopenia - Proposed hypothesis

To further investigate other biological explanations for elevated PAIgG in patients with ITP and non-immune thrombocytopenia, I utilized ultrastructural techniques including, laser scanning confocal microscopy and transmission electron microscopy as a novel investigative approach to the study of PAIgG. I hypothesized that by characterizing the immunomorphologic characteristics of PAIgG in platelets from patients with ITP and non-immune thrombocytopenia information regarding the subcellular distribution of IgG may provide alternate explanations for why IgG-sensitized platelets are destroyed in one thrombocytopenic disorder (ITP) and not in another (non-immune thrombocytopenia). Currently, there is little information on the use of diagnostic electron microscopy in the field of platelet pathology and no information on the immunomorphologic characteristics of PAIgG in thrombocytopenic disorders.

Results of this thesis project demonstrated that platelets from patients with ITP and non-immune thrombocytopenia are morphologically normal but differ in their immunomorphologic characteristics of PAIgG. More specifically, PAIgG in platelets from patients with ITP is primarily localized within α-granules with much smaller amounts on the platelet surface and in channels of the open-cannalicular system. In comparison, PAIgG in platelets from patients with non-immune thrombocytopenia is localized almost entirely within α-granules with an absence of IgG on the

platelet surface or within the open-cannalicular system. This pattern of PAIgG distribution is consistent for intact fixed platelets (analyzed by confocal microscopy and flow cytometry), isolated platelet membrane fractions (analyzed by serologic assay) and ultra-thin platelet sections (analyzed by electron microscopy). Regardless of the technique applied and irrespective of elevated total PAIgG levels, platelets from patients with non-immune thrombocytopenia failed to demonstrate elevated amounts of IgG on the platelet surface. This observation differed from that reported previously (Kelton *et al*, 1982; 1983; 1985; George, 1989) and suggested that either previous serologic measurements of surface PAIgG in patients with non-immune thrombocytopenia were falsely elevated or that ultrastructural evaluation of surface PAIgG in platelets from patients with non-immune thrombocytopenia is falsely negative.

In previous investigations surface PAIgG was routinely measured using direct binding assays. However because of the unique characteristics of the platelet membrane precise measurement of antibody on the surface of platelets is difficult (Warner and Kelton, 1997). This difficulty is reflected in the enormous variation in reported surface PAIgG values for normal platelets (between 100 to several thousand molecules of IgG per platelet) (Kelton *et al*, 1989). One possible explanation for the observed variation may be due to differences in the handling of platelets before analysis (Sinha and Kelton, 1990). Moreover, it is suggested that differences in the platelet processing technique used in this thesis project versus previous investigations may account for the observed absence of IgG on the surface of platelets from patients with non-immune thrombocytopenia. In direct binding assays platelets are routinely isolated by differential centrifugation with multiple washes and often sit overnight prior to analysis. In the present investigation unintentional platelet activation was minimized by routinely processing platelets in a specific manner. Firsty, whole blood was collected

into an anticoagulant containing a cocktail of platelet activation inhibitors. Secondly, platelets were processed immediately (within one hour) following blood collection. Thirdly, platelets were isolated by the less activating method of gel-filtration rather than differential centrifugation (Wencel-Drake *et al*, 1996; Simmons and Albrecht, 1996). It is hypothesized that these platelet processing steps were crucial in obtaining resting platelets as validated by morphological examination and measurement of a platelet activation marker. It is further hypothesized that previous measurements of elevated surface PAIgG in platelets from patients with non-immune thrombocytopenia may have resulted from *in vitro* leakage of α-granule IgG onto the surface of platelets over time. In this way the time-dependent appearance of IgG on the surface of these platelets may reflect the elevated amount of PAIgG within these platelets rather than the amount of true IgG on the surface of platelets (Pfueller and David, 1986). In these investigations platelets from patients with non-immune thrombocytopenia when processed immediately demonstrated an absence of IgG staining on the platelet surface. However, when there was a deliberate delay in processing and platelets were allowed to sit overnight prior to analysis, focal points of IgG were observed on the platelet surface. This pattern of IgG distribution was also observed in platelets from a group of thrombocytopenic patients (non-immune) in which there was an unavoidable delay in processing (whole blood sat overnight before platelets were isolated). The results of these investigations suggest that *in vitro* leakage of elevated amounts of PAIgG within platelets onto the platelet surface may result in serologic measurement of elevated surface PAIgG. Observations that immunomorphologic characteristics of surface PAIgG differ in patients with immune and non-immune thrombocytopenia suggest that rim patterns of surface PAIgG in patients with ITP reflect the binding of true autoantibodies to glycoprotein targets on the platelet surface whereas, "beaded necklace" patterns of surface PAIgG in patients with non-immune thrombocytopenia reflect the appearance of IgG

exocytosis from internal storage pools. These results provide further insight into the biological explanation of how IgG-sensitized platelets in patients with non-immune thrombocytopenia escape premature clearance by phagocytic cells of the reticuloendothelial system. In these patients it is hypothesized that platelets are not prematurely cleared because IgG is not elevated on the platelet surface. These results further explain why patients with non-immune thrombocytopenia have a normal platelet lifespan. Finally, although the subcellular distribution of surface PAIgG in patients with ITP and non-immune thrombocytopenia is different, the mechanism by which IgG (that is not bound to antigen) accumulates within these platelets may be similar.

Further investigation of immunomorphologic characteristics of surface PAIgG in ITP patients indicated that there may be at least two subsets of patients. In the majority of ITP patients anti-GPIIb/IIIa or anti-GPIb/IX IgG autoantibodies were demonstrated in platelet lysate using an antigen capture assay. In these patients rim pattern staining of IgG on the platelet surface was observed. In a second smaller group of ITP patients (n=5), anti-GPIIb/IIIa or anti-GPIb/IX IgG autoantibodies were not detected in platelet lysate. In these patients IgG staining on the surface of platelets was not observed. This absence of staining was not attributable to degree of thrombocytopenia (which was similar for both groups) or obvious clinical differences between the two ITP patient groups. However, there are several limitations to these studies. Firstly, the number of ITP patients studied with negative antigen capture results was small (n=5). Since ITP is a diagnosis of exclusion, it is possible that some patients with negative antigen capture results and negative IgG rim staining patterns have thrombocytopenic disorders other than ITP. Secondly, the immunormorphologic characteristics of only platelet IgG and not platelet IgM was studied. It is possible some ITP patients have platelet destruction mediated primarily by IgM anti-platelet autoantibodies which would not

have been detected. Thirdly, ITP patients with rim pattern staining all had IgG anti-platelet autoantibodies directed against common platelet targets (GPIIb/IIIa and GPIb/IX) as determined by an antigen capture assay. Therefore rim pattern staining in these patients represented IgG antibody bound in increased amounts on the platelet surface. In ITP patients with no rim pattern staining it is possible that IgG autoantibodies were directed against targets that are present in lower copy numbers on platelet membranes, such as GPIa-IIa or GPIV (van Leeuwen *et al*, 1982) which were not measured in our antigen capture assay. Binding of IgG antibodies to less common platelet targets may have appeared as negative IgG staining on the platelet surface while triggering similar accelerated platelet clearance. It is suggested that further investigation of larger numbers of ITP patients with negative antigen capture results is necessary to validate these hypotheses.

6.4 Proposed mechanism by which IgG accumulates in platelets

Observations of an isolated increase in the exogenous α-granule protein content in thrombocytopenic patients suggests abnormalities in the pathway of plasma protein acquisition. For many years it was shown that platelets were capable of sequestering a wide variety of particulate and soluble material within channels of the surface-connected canalicular system (Movat *et al*, 1965; White, 1968; Zucker-Franklin, 1981). More recently there is evidence to suggest that megakaryocytes and/or platelets have the ability to internalize and incorporate exogenous proteins directly into secretory α-granules (Handagama *et al*, 1989). The ability of cells to acquire proteins into secretory granules by endocytosis has rarely been reported and is not described in several reviews of endocytosis and protein secretion (Kelly and Burgess, 1987; Klausner, 1989; Lingappa, 1989; Van Deurs *et al*, 1989; Schwartz, 1990).

In this thesis project the immunomorphologic characteristics of PAIgG in platelets incubated with immune (anti-GPIIb/IIIa antibody) and non-immune IgG provide further information about possible pathways of IgG internalization in platelets. The observation that IgG is increased in α-granules over time following incubation of normal platelets with immune (anti-GPIIb/IIIa antibody) but not non-immune IgG suggests platelet pathways of IgG internalization are specific. One possible pathway of internalization may involve a process of receptor-mediated endocytosis by binding to the GPIIb/IIIa complex. This complex has been shown to actively cycle from the platelet surface to internal storage pools in resting platelets and may be important for clearing the platelet surface of specific anti-platelet antibody (Wencel-Drake, 1990; White *et al*, 1996). In normal platelets GPIIb/IIIa has been found to be associated with the external side of the plasma membrane, the intraluminal face of the open-cannalicular system and the internal face of the α-granule membrane (Cramer *et al*, 1990). This pattern of glycoprotein distribution was identical to the distribution of IgG in platelets incubated with immune IgG (anti-GPIIb/IIIa antibody). These observations suggest that specific anti-GPIIb/IIIa antibody may be routed to platelet α-granules via a membrane-bound pathway. However, this pathway does not adequately explain why large amounts of IgG and other plasma proteins including albumin, are found within α-granules. Incubation of normal resting platelets with large concentrations of non-specific IgG alone is not adequate for IgG uptake. This observation suggests that either other factors such as longer incubation periods or platelet activation are important for uptake of non-specific IgG. Experimental studies have investigated the non-specific binding of plasma proteins to platelets following an initial activation event. In *in vitro* studies by Winiarski (1985) the binding of heat aggregated IgG to the surface of platelets resulted in increased amounts of IgM, IgA and albumin on the platelet surface. Similar results were also reported by Sinha *et al* (1988) using an animal model of immune thrombocytopenia. In these *in vivo* studies,

infusion of guinea pig serum into rabbits caused a dose-dependent thrombocytopenia, paralleled by an increase in guinea pig IgG, rabbit IgG and rabbit albumin on the platelet surface. These results were interpreted as indicating that the initial binding of platelet-specific antibody to the platelet surface resulted in the subsequent binding of non-specific IgG and other plasma proteins. In investigations in this thesis project, the addition of immune or non-immune IgG to normal resting platelets did not result in platelet activation as indicated by measurement of a platelet activation marker (P-selectin). Although the subsequent binding and endocytosis of plasma proteins such as albumin were not analyzed in these investigations, the results of my studies suggest that platelet activation was not necessary for the uptake and transport of anti-GPIIb/IIIa antibody to α-granules.

Finally, mechanisms other than platelet endocytosis may be responsible for plasma protein uptake in thrombocytopenic patients. One such mechanism would be the stimulation of fluid-phase endocytosis by megakaryocytes. This mechanism could explain why PAIgG is elevated within platelets of patients with thrombocytopenia due to diverse causes without a parallel increase in endogenous α-granule proteins.

6.5 Nature of PAIgG and clinical usefulness of PAIgG assays

The results of this thesis project provide further information regarding measurements of PAIgG using phase II assays. The observation that IgG is elevated in parallel with other plasma proteins (albumin and fibrinogen) in thrombocytopenic patients suggests that measurements of PAIgG and measurements of any other plasma-derived alpha granule protein (such as platel*et al*bumin) are not able to discriminate ITP from other thrombocytopenic states. Similarly, immunomorphologic characterization of PAIgG within platelets provides no useful diagnostic information. In these

studies, platelets from all patients studied including: (1) patients with immune and non-immune thrombocytopenia, (2) patients with normal platelet counts, and (3) ITP patients with positive and negative antigen-capture results demonstrated identical granular patterns of IgG staining. These staining patterns were indistinguishable from the staining patterns of endogenous α-granule proteins such as multimerin, and other exogenous α-granule proteins such as albumin. Hence, measurement of total PAIgG or analysis of patterns of IgG staining within platelets is not useful in the differential diagnosis of thrombocytopenic disorders.

The observation that elevated PAIgG is observed in patients with diverse clinical etiologies (both immune and non-immune) suggests that increased total PAIgG may be unrelated to platelet clearance. The pathologically important IgG was thought to be associated with the platelet surface. For this reason, observations that surface PAIgG was elevated in patients with both immune and non-immune thrombocytopenia were confusing. Results from this thesis project demonstrated that this observation may not be entirely correct. In these studies, I demonstrated that IgG is absent on the surface of platelets from patients with non-immune thrombocytopenia but may occur over time as a result of *in vitro* leakage of α-granule IgG onto the surface of platelets. These observations questioned the origin of surface PAIgG measured in previous reports using serologic assays. Observations that immunomorphologic characteristics of surface PAIgG in patients with ITP versus non-immune thrombocytopenia differ suggest that it may be possible to categorize thrombocytopenic patients based on patterns of platelet surface IgG distribution. Hence, IgG rim pattern staining may be as diagnostically meaningful as positive antigen capture results.

6.6 Significance of results

Currently, ultrastructural studies are primarily used in the differential diagnosis of inherited platelet disorders with little to no information available for thrombocytopenic disorders. In inherited platelet disorders certain platelet structural defects can be characteristic. For example, platelets from patients with gray platelet syndrome (GPS) are markedly deficient in morphologically recognizable platelet α-granules whereas platelets from patients with platelet dense granule storage pool deficiency (SPD) disorder are almost completely devoid of dense bodies (White, 1998). In patients with αδ-storage pool deficiency there is a deficiency of both dense granules and α-granules and in patients with Paris-Trousseau thrombocytopenia platelets contain enlarged α-granules (Smith *et al*, 1997). In other congenital disorders electron microscopy has been used to differentiate among the giant platelet disorders including; May-Hegglin anomaly (MHA) (White, 1998), Fechtner syndrome (Peterson, 1985), Epstein's syndrome (Epstein, 1972), and Sebastian's syndrome (Young *et al*, 1999). In this heterogenous group of macrothrombocytopenias certain morphological data such as the presence or absence of leukocyte inclusions and the ultrastructure of the inclusions themselves, have proven useful in differentiating one disorder from another (Young *et al*, 1999; White, 1998). This thesis describes the novel use of morphological data to differentiate between platelets from patients with immune and non-immune thrombocytopenia. The results of these studies demonstrate that evaluation of platelet ultrastructure alone is not useful in differentiating patients with immune and non-immune thrombocytopenia but analysis of immunomorphologic characteristics of PAIgG may provide additional information. In this way, surface PAIgG distribution patterns may prove useful for the differential diagnosis of immune thrombocytopenic disorders and in the initial diagnosis of asymptomatic ITP patients. Additionally, it may be possible to further categorize ITP patients based on patterns of IgG distribution or patterns of IgM distribution. These patterns by themselves or in

conjunction with other laboratory measures may identify subsets of patients with antibodies directed against platelet targets other than known glycoproteins. Finally, the results of this thesis project refute a currently accepted concept that platelets in patients with ITP are larger than normal with increased α-granule content. These studies provide evidence of quantitative abnormalities of platelet proteins restricted to the pool of exogenous α-granule proteins with no apparent disruption of normal platelet function. Observations of accumulation of platelet-specific IgG in α-granules over time provides additional evidence that abnormalities in the pathway of plasma protein accumulation in patients with ITP may involve mechanisms of platelet endocytosis.

6.7 Implications for future research

The autoimmune nature of ITP is well established, however the diagnostic criteria which require exclusion of other thrombocytopenic disorders,do not include identification of platelet autoantibodies (Winiarski, 1998). Despite the fact that phase II PAIgG assays are being increasingly replaced by more diagnostically meaningful phase III assays (antigen capture assays) recently published practice guidelines developed for the American Society of Hematology (George *et al*, 1996) deemed the use of phase II or phase III PAIgG assays as unnecessary in the routine evaluation of adult and childhood ITP. Currently a reliable diagnostic test for ITP is not available. Investigations in this thesis project suggest that morphologic evaluation of platelets may prove to be diagnostically useful to distinguish immune from non-immune thrombocytopenia. It is suggested that this new methodology may reshape the diagnostic criteria for ITP and the current view on elevated surface PAIgG in immune and non-immune thrombocytopenia. Further investigation of immunomorphologic characteristics of PAIgG and PAIgM in patients with thrombocytopenic disorders are necessary in understanding why some ITP patients are negative in antigen capture

assays. It is hypothesized that ultrastructural techniques may allow for better classification of subsets of ITP patients with improved approaches to treatment and better predictive capability of those patients more likely to suffer life-threatening hemorrhage.

The mechanisms behind elevated PAIgG and its role in relation to platelet kinetics have not been fully explained. Whereas, investigations in this thesis focused on platelets alone, questions regarding the role of the megakaryocyte in the uptake of plasma proteins were not addressed. Further investigation of the immunomorphologic characteristics of PAIgG in megakaryocytes of patients with immune and non-immune thrombocytopenia is necessary to further evaluate possible mechanisms of IgG accumulation. It is hypothesized that these investigations will contribute to the biological explanation for why exogenous α-granule proteins are abnormally accumulated in platelets from patients with a variety of thrombocytopenic disorders. These investigations may also provide additional insight into the functional significance of elevated protein concentrations in platelets.

Lastly, aspects of protein trafficking in platelets have not been fully elucidated. The ultrastructural techniques utilized in this thesis project offer a convenient and useful approach to the further investigation of aspects of platelet endocytosis. Through further immunomorphologic characterization of IgG and other plasma-derived platelet proteins in ITP patients with antibodies directed against specific glycoprotein targets, aspects of antibody clearance from the platelet membrane can be addressed.

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APPENDIX 1

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Morphological analysis of microparticle generation in heparin-induced thrombocytopenia

Mary Hughes, Catherine P. M. Hayward, Theodore E. Warkentin, Peter Horsewood, Katherine A. Chorneyko, and John G. Kelton

Heparin-induced thrombocytopenia (HIT) with thrombosis is a serious complication of heparin use. HIT sera can generate platelet-derived microparticles, which are produced in a heparin-dependent manner and are hypothesized to be important initial pathological participants because they promote vascular occlusion. To date, microparticles have been studied using flow cytometric techniques. However, it is uncertain whether the small-sized material seen in flow cytometric studies represents true platelet microparticles shed from activated platelets or whether they are platelets that have contracted after releasing their internal components. This report describes a morphological investigation of platelet-derived microparticles in HIT using, among other techniques, confocal, scanning electron, and transmission electron microscopy. Following incubation with HIT sera, the existence of small membrane-bound vesicles in the milieu of activated platelets was demonstrated. A population of microparticles, expressing platelet-specific glycoproteins, was separated from platelets by centrifugation over a sucrose layer. These microparticles had identical flow cytometric profiles, size heterogeneity, and GPIb$_\alpha$ and GPIIb/IIIa staining intensity as the microparticle population in unfractionated samples. When microparticles were generated in situ and fixed onto grids, they were demonstrated to be distinct membrane-bound vesicles that originated near the platelet body and terminal ends of pseudopods on activated platelets. These microparticles appeared to be generated by localized swelling, budding, and release. Collectively, these morphological studies document the existence of true microparticles in platelets activated by HIT sera. The microparticles may play an important role in the pathogenesis of HIT. (Blood. 2000;96:188-194)

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Introduction

Heparin-induced thrombocytopenia (HIT), one of the most common immune-mediated adverse drug reactions, occurs in 1%-3% of patients receiving therapeutic doses of heparin.[1,2] Unlike other immunological drug-induced reactions, many patients with HIT experience serious thrombotic morbidity including venous thrombosis, arterial thrombosis, and disseminated intravascular coagulation (DIC).[1-3] Studies of the pathogenesis of this prothrombotic condition demonstrated that circulating heparin-dependent antibodies bind to a complex of heparin and platelet factor 4 (PF4).[4-8] Several years ago, we demonstrated that the binding of heparin-dependent immunoglobulin G (IgG) to platelet FcγRII receptors leads to platelet activation[9,10] and the generation of platelet-derived microparticles.[11] These microparticles were produced in a heparin-dependent fashion and were shown to have procoagulant activity.[11,12] Although microparticles are thought to be initial participants in venoocclusive events, their mechanism of formation and confirmation of existence are still a matter of controversy.

Presently, flow cytometry is the most frequently reported technique used to study platelet-derived microparticles, and it has been used by our group to develop a diagnostic test for heparin-induced thrombocytopenia.[13] However, some investigators have raised questions about the analysis of flow cytometry experiments. Studies by Bode et al[14] showed that the light scatter distribution of platelets is broad and that it is difficult to identify clearly where the population of intact platelets ends and the population of smaller particles begins. This observation of a continuum of particle size rather than 2 distinct platelet and microparticle populations questioned the nature of microparticles and their degree of heterogeneity. Studies by Matzdorff et al[15] demonstrated that counting microparticles becomes unreliable when platelet counts drop to a low number. This was thought to be explained by the fact that saturating amounts of antibodies may become unspecifically adsorbed to other particles or may form antibody complexes. The observations that an antibody surplus leads to antibody complexes and that these complexes can interfere with platelet and microparticle counting have also been reported by other groups.[16,17] Finally, George et al[18] demonstrated that microparticle preparations derived from washed activated platelets contained a heterogeneous array of membrane fragments, vesicles, and granules. These observations suggested that morphological documentation of microparticles in heparin-induced thrombocytopenia was necessary for the confirmation of their existence.

In previous studies, immunoassays and flow cytometry studies demonstrated procoagulant properties of microparticles but failed to provide information on their structural origin or mechanism of generation. In this study we conducted a morphological analysis of platelet-derived microparticles to document the existence of microparticles in heparin-induced thrombocytopenia. Several different

From the Departments of Medicine and Pathology, Faculty of Health Sciences, McMaster University Medical Center, and The Canadian Blood Services, Hamilton, Ontario, Canada.

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