J.J.E. VOS

PLATELET-ASSOCIATED IgG and IDIOPATHIC THROMBOCYTOPENIC PURPURA

PLATELET-ASSOCIATED IgG AND IDIOPATHIC THROMBOCYTOPENIC PURPURA

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van
doctor in de Geneeskunde
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
dr. D.W. Bresters,
hoogleraar in de Faculteit
der Wiskunde en Natuurwetenschappen
in het openbaar te verdedigen
in de Aula der Universiteit
(tijdelijk in het Wiskundegebouw,
Roetersstraat 15)
op
donderdag 19 juni
om 17.00 uur

door

JAN JANS ELING VOS

geboren te Groningen

1986

Offsetdrukkerij Kanters B.V.,
Alblasserdam

Promotor : Prof. dr. C.P. Engelfriet

Co-promotores : Dr. J.G. Huisman

: Dr. A.E.G. Kr. von dem Borne

Beoordelingscommissie : Prof. dr. R. Goudsmit

Prof. dr. M.M.A.C. Langenhuijsen

Prof. dr. J.J. Sixma

This thesis has been prepared in the departments of Developmental Research (head: Dr. C. Aay) and Immunohaematology (head: Prof. dr. C.P. Engelfriet) at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (research director: Prof. dr. V.P. Eijsvoogel) and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands.

I express my gratitude to all who have contributed to this thesis.

The manuscript was typed by Mrs J. Gerritsen for which I am very grateful.

Cover: M.S. Bijleveld

ABBREVIATIONS

AIDS	-	acquired immune deficiency syndrome
AIHA	-	autoimmune haemolytic anaemia
AITP	-	autoimmune thrombocytopenic purpura
BSA	-	bovine serum albumin
CLB	-	Centraal Laboratorium voor de Bloedtransfusiedienst van het Nederlandse Rode Kruis
ELISA	-	enzyme-linked immunosorbent assay
FITC	-	fluorescein isothiocyanate
GP	-	glycoprotein
HA	-	hypoplastic anaemia
HIgG	-	human IgG
HLA	-	human leukocyte antigens
IFT	-	immunofluorescence test
Ig	-	immunoglobulin
ITP	-	idiopathic thrombocytopenic purpura
IVG	-	intravenous gammaglobulin
kD	-	kilo Dalton
McAb	-	monoclonal antibody
MPS	-	mononuclear phagocytic system
NEM	-	N-ethylmaleamide
NP40	-	Nonidet P40
PAGE	-	polyacrylamide gelelectrophoresis
PA-Ig	-	platelet-associated Ig
PB-Ig	-	platelet-bound Ig
PBS	-	phosphate-buffered saline
PFA	-	paraformaldehyde
P(S)IFT	-	platelet (suspension) immunofluorescence test
PIFT-IgG	-	PIFT performed with anti-IgG
PRP	-	platelet-rich plasma
RA	-	radioactivity; rheumatoid arthritis
RIA	-	radioimmunoassay
SCCS	-	surface-connected canalicular system
SDS	-	sodium dodecyl sulfate
SLE	-	systemic lupus erythematosus
STP	-	secondary thrombocytopenia
TNT	-	buffer containing Tris-HCl (0.05 M), NaCl (0.15 M) and Tween-20 (0.05% v/v)

CONTENTS

CHAPTER I

PLATELET-ASSOCIATED IgG AND IDIOPATHIC THROMBOCYTOPENIC PURPURA: A SHORT REVIEW

This thesis deals with some aspects of the diagnosis and treatment of idiopathic thrombocytopenic purpura (ITP).

Idiopathic thrombocytopenia is generally considered to be a disease caused by circulating anti-platelet factors (1), probably autoantibodies directed against platelet antigens. Therefore, this disease is also called autoimmune thrombocytopenia (AITP).

In the last few years, increasing evidence for this mechanism has been provided by the demonstration of increased levels of platelet-associated or -bound immunoglobulins and by characterization of the antigens on the platelet surface, possibly acting as targets for these autoantibodies.

Pathogenesis, diagnosis and therapy of ITP have been extensively reviewed (2-4). In addition to these reviews, some important new insights in the origin of platelet-associated immunoglobulins and their relationship to ITP will be discussed in this Chapter.

Diagnosis

The diagnosis chronic ITP is made by exclusion of other causes of thrombocytopenia. The disease is defined as a thrombocytopenia (platelet count $<150 \times 10^9/1$) without splenomegaly at physical examination, with a normal bone marrow with adequate or abundant numbers of megakaryocytes and a normal blood coagulation status, except for a prolonged bleeding time, whereas other causes of thrombocytopenia have been excluded by clinical features and laboratory investigations (2-4).

The only clinical symptoms are mostly easy bruising and in women menoragghia. At physical examination, petechiae and ecchymoses may be found. Other abnormalities, such as lymphadenopathy or hepatosplenomegaly, suggest another diagnosis or an associated disease. In severe thrombocytopenia, mucosal bleeding may occur and, very rarely, intracranial bleeding often with a fatal outcome. However, quite often thrombocytopenia is asymptomatic and symptoms are present, mostly only at platelet counts lower than $40 \times 10^9/1$.

Haematological tests show normal values, except for the platelet count.

Bleeding time may be prolonged.

In ITP, platelet survival is always shortened and is directly correlated with the platelet count (2-4). The increased destruction of the labelled platelets is believed to be caused by rapid platelet destruction by the mononuclear phagocytic system (MPS, i.e. spleen, liver and bone marrow).

Immunclogical investigations

Autoimmunity is thought to play a central role in the pathogenesis of ITP. The importance of cell-mediated immunity has not been very well established yet (2-4). The role of humoral immunity in ITP has been studied extensively. Harrington et al. (1) provided in 1951 proof for the autoimmune character of ITP by demonstrating a transmissable anti-platelet factor in patients' sera, causing thrombocytopenia in normal healthy donors.

Initially, the many attempts to detect platelet autoantibodies were not very successful, probably because of the following reasons. First, normal platelets appeared to have a large amount of 'background' IgG in contrast to e.g. erythrocytes, probably because of their spongy nature (surface-connected canalicular system). Moreover, the amount of IgG necessary for platelet destruction would be expected to be very small, considering the amounts of IgG needed for erythrocyte destruction (5). Second, until the Seventies many assays for the detection of platelet antibodies depended on their biological function (agglutination, complement fixation) or were qualitative, leading to often unpredictable results.

In the last decade, numerous assays that quantitate platelet-associated immunoglobulins (PA-Ig) have been described, circumventing the above problems: radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA) and assays employing radioimmuno diffusion (2,3). In these assays, the amount of IgG in a washed platelet suspension (determined by comparison with known amounts of purified immunoglobulins) is related to the number of platelets known to be present in the test sample. In this way, PA-Ig can be quantitated.

Most assays measure only PA-IgG. PA-IgG measured in normal donor platelets as well as in platelets of thrombocytopenic patients differs considerably as reported by various authors (normal donors: 0.1-26 fg IgG/platelet; ITP patients: 0.1-1800 fg IgG/platelet). These PA-IgG values in normal donors are 10 to 20 times higher than expected when PA-IgG is estimated from the number of platelet Fcγ-receptor molecules (0.1-0.5 fg IgG/platelet) (6). Moreover, amounts of PA-IgG are measured on platelets of patients that could hardly fit on the platelet surface, because of limitations in the number of available antigenic determinants.

Another assay for detecting platelet-bound IgG is the platelet immunofluorescence test (PIFT; 7). This technique has the advantage that only intact platelets are examined by which the influence on PA-IgG measurement of platelet fragments and/or aggregates are excluded. Moreover, platelet-bound IgG, IgM and IgA can be detected by this technique. In our laboratory, a great deal of experience has been gained in employing this technique.

Some remarks on the measurement of platelet-associated immunoglobulins

Because most assays measure only PA-IgG, mainly influences on PA-IgG measurement are discussed.

Technical influences

Naturally, it is important to wash platelets thoroughly since the expected amount of PA-IgG is approximately a thousandfold lower than the amount of IgG in the environment.

The choice of the anticoagulant is important. Ethylenediamine tetra acetate (EDTA) prevents platelet aggregation excellently (see ref. 3), while introducing detection of so-called EDTA-dependent antibodies and the EDTA phenomenon, i.e. *in-vitro* platelet agglutination due to EDTA-dependent antibodies (8). The use of a fixative, e.g. paraformaldehyde (PFA), can lead to detection of PFA-dependent antibodies (8).

Moreover, it has been suggested that direct assays measure lower PA-IgG values than consumption assays, because of differences in affinity of the applied anti-IgG for platelet-bound IgG compared to 'free' IgG, used as reference standard in consumption assays (9).

Finally, platelet solubilization or sonication before PA-IgG measurement leads to the detection of higher amounts of PA-IgG, because intracellular IgG is being liberated from the platelets and measured as well (10) (see Fig. 1).

Platelet-specific influences (Fig. 1)

Non-immune or non-specific PA-IgG

Various factors have to be considered to explain the existence of non-

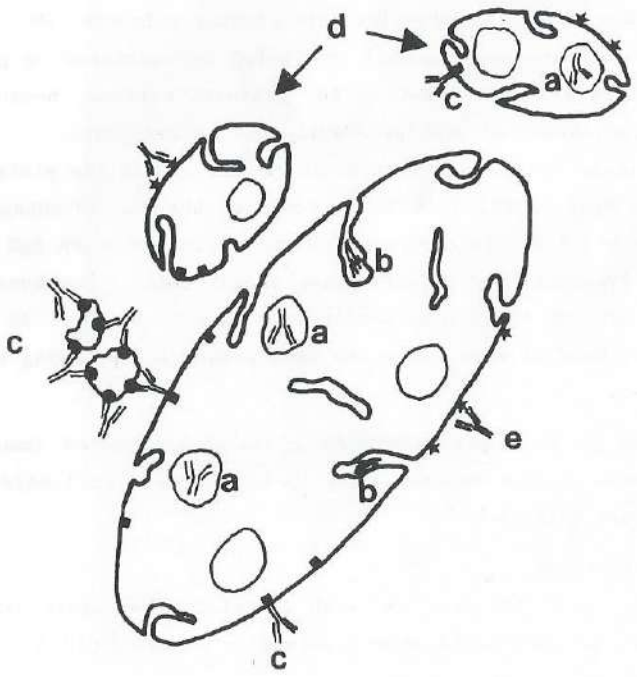

Fig. 1 - Locations of platelet-associated IgG. a) Intracellular: probably located in α-granules. b) In the surface-connected canalicular system (SCCS). c) Bound to Fcγ receptor (■) as monomeric Ig or as immune complexes. d) On platelet fragments or (not drawn) platelet aggregates. These particles cannot be accurately counted by electronic particle counters and therefore, a falsely elevated PA-IgG value may be measured. e) Specifically bound to target antigens on the platelet surface (★).

immune or non-specific PA-IgG. First, platelets have been demonstrated to react specifically with one particular subdivision of human plasma IgG (11). This non-immune IgG might be linked to the cellular aging process and might trigger the removal of senescent platelets, similarly as has been described for erythrocyte clearance (12). It is still unclear what exactly defines an 'old' platelet. Platelet age has been related to platelet density and platelet size (for review see ref. 13). The preparation of an 'old' platelet population remains difficult and a possible relationship between non-immune PA-IgG (as has been suggested by various authors) and platelet age is still controversial.

PA-IgG might also be plasma IgG entrapped by platelets, as suggested by experiments of Kelton et al. (14). A possible site for IgG entrapment is the so-called platelet surface-connected canalicular system, because of its direct connection with the platelet surroundings (15,16).

Furthermore, PA-IgG may be immune complexes bound by the platelet Fcγ receptor (17-19). Elevated PA-IgG levels are indeed found in diseases with increased amounts of circulating immune complexes (20,21). An increased Fcγ-receptor expression, as has been described for myeloproliferative diseases (22), might lead to increased PA-IgG levels.

In states of increased platelet destruction and/or platelet aggregation, circulating platelet fragments respectively aggregates may develop. Platelet aggregation may also occur *in vitro* due to the so-called EDTA phenomenon (23). Accurate PA-IgG measurement might be hindered in these cases, because platelet fragments and aggregates contribute to the amount of (platelet-associated) IgG in the test suspension, while they are not detectable in the Coulter counter, leading to falsely elevated PA-IgG levels (24).

Finally, in diseases with an abnormally functioning mononuclear phagocytic system (MPS), platelets sensitized with antibodies, which would otherwise be eliminated, remain in the circulation, thus increasing PA-IgG (25).

Influences on PA-IgG determination, such as storage of blood samples and the presence of platelet fragments, will be dealt with in Chapter II. For this purpose, a recently developed radioimmunoassay for the detection of PA-IgG was used. Also, in this Chapter, the usefulness of this test to quantify platelet-bound antibodies will be demonstrated by measurements on phenotyped donor platelets, sensitized by well-defined antisera.

In Chapter III, the results with the platelet immunofluorescence test (PIFT) in a consecutive series of 255 thrombocytopenic patients are described. Results of PIFT and our radioimmunoassay for PA-IgG are compared in another series of 130 thrombocytopenic patients, which is described in Chapter IV. Similarities and discrepancies found between the two tests will be discussed. The occurrence of IgM antibodies (as determined by PIFT) and platelet fragments (as determined by Coulter counter S plus II) as causes of discrepancies will be discussed.

In Chapter V, a technique for the detection of circulating platelet

fragments is described which could demonstrate the occurrence of such fragments in thrombocytopenic patients. With this technique, we were able to monitor platelet contamination in filtered red blood-cell preparations. Results of this study are described in Chapter VI.

Targets for 'immune' ('specifically bound') PA-IgG

Specific binding of platelet antibodies has been made plausible by elution experiments (26). The lack of reactivity of most platelet autoantibodies with platelets from patients with Glanzmann's disease type I (lacking the GP IIb-IIIa complex) suggested that the platelet GP IIb-IIIa complex might be target for most platelet autoantibodies. Other studies using radioimmunoassays on purified platelet glycoproteins have demonstrated specific reactivity of some sera containing platelet autoantibodies with GP IIb-IIIa and with GP Ib (27,28).

Recently, immunoprecipitation (29) and immunoblotting (30) have been used for the identification of platelet-specific antigens. Some sera containing autoantibodies could precipitate the GP IIb-IIIa complex (MW 134-140 kD and 89-105 kD, respectively), whereas in immunoblotting a small number of sera reacted only with a 89-105-kD band (GP IIIa). Reactivity of autoantibodies containing sera with bands of 220-250 kD has also been observed (29). These results suggested that GP IIb, GP IIIa and/or the GP IIb-IIIa complex might be specific targets for platelet autoantibodies. However, the reactivity in both techniques has to be interpreted carefully, because reactivity might be due to non-specific protein-protein interactions. Moreover, a negative reaction in either technique does not necessarily mean that a platelet autoantigen is not located on a particular protein, because conformational determinants of the antigens may be lost during platelet preparation and solubilization before gel electrophoresis.

In Chapter VII, some preliminary observations on reactivity of sera containing autoantibodies, alloantibodies or antibodies directed against so-called cryptantigens are reported using the immunoblotting technique.

Therapy

Childhood ITP is initially treated expectively, unless severe bleedings are present. In adult ITP, treatment is started as soon as the diagnosis has been established with the primary objective to obtain 'safe' platelet counts (above $40 \times 10^9/l$).

High doses of corticosteroids (1 mg/kg body weight/day) are the first-choice therapy. If no remission is obtained, splenectomy should be done. In this way complete remissions can be obtained in 80-90% of the patients. When splenectomy fails, other treatments may be tried: immunosuppressive agents (azathioprine, cyclophosphamide, vinca alkaloids or vinblastine-loaded platelets) and danazol (31).

Recently, high-dose intravenous gammaglobulin (IVG) has been reported as a new therapeutic agent for ITP (32). In a large number of patients, IVG has been able to produce a fast rise of the platelet count, mostly transiently. Therefore and because of its high costs, this form of therapy should be reserved for emergency treatment when intracranial or large gastro-intestinal bleedings occur.

The results of a study on the effect of high-dose intravenous gammaglobulin therapy with a preparation produced in our laboratory are described in Chapter VIII. In this Chapter, some parameters (strength of PIFT, C1q-binding assay, splenectomy) were related to the response to IVG treatment. The working mechanism of IVG treatment is not yet clear. The most plausible explanations so far are inhibition of Fcγ receptor-mediated platelet destruction and inhibition of autoantibody synthesis (33-35). For the last hypothesis, the following model may exist. Immunoglobulin administration leads to generation of immunoglobulin-binding factors by binding of immunoglobulins to shedded Fc-receptor molecules. These complexes induce then B- and/or T-cell suppression by which autoantibody synthesis is inhibited (36; not referred to in Chapter VIII).

REFERENCES

1. Harrington WJ, Mimich V, Hollingsworth JW, Moore CV (1951) Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J Lab Clin Med 38: 1-10.
2. McMillan R (1981) Chronic thrombocytopenic idiopathic purpura. New Engl J Med 304: 1135-1147.
3. Borne AEG Kr von dem (1984) Autoimmune thrombocytopenia. In: Engelfriet CP, van Loghem JJ, von dem Borne AEG Kr (eds) Immunohaematology. Elsevier, Amsterdam, pp 222-256.
4. Karpatkin S (1985) Autoimmune thrombocytopenic purpura. Sem Hematol 22: 260-288.
5. Constantoulakis M, Costea N, Schwartz RS, Dameshek W (1963) Quantitative studies of the effect of red blood cell sensitization on *in-vitro* haemolysis. J Clin Invest 42: 1790-1801.

6. Karas SP, Rosse WF, Kurlander RJ (1982) Characterization of the IgG-Fc receptor on human platelets. Blood 60: 1277-1282.

7. Borne AEG Kr von dem, Helmerhorst FM, Leeuwen EF van, Pegels JG, Riesz E von, Engelfriet CP (1980) Autoimmune thrombocytopenia: detection of platelet autoantibodies with the suspension immunofluorescence test. Brit J Haematol 45: 319-327.

8. Pegels JG, Borne AEG Kr von dem, Bruynes ECE, Engelfriet CP (1982) Pseudothrombocytopenia: an immunological study on platelet antibodies dependent on ethylene diamine tetra-acetate. Blood 59: 157-161.

9. Rosse WF, Devine DV, Ware R (1984) Reactions of immunoglobulin G-binding ligands with platelets and platelet-associated immunoglobulin G. J Clin Invest 73: 489-496.

10. Kelton JG, Denomme G, Gauldie J (1980) The majority of platelet-associated IgG (PA-IgG) is in the interior of platelets and not measurable using standard assays. Transfusion 201: 625-626.

11. Steiner M (1985) Platelet-associated IgG is a specific protein. Biochem Biophys Res Commun 129: 206-212.

12. Aldeman EM, Fudenberg HH, Lovins RE (1981) Isolation and characterization of an age-related antigen present on senescent human red blood cells. Blood 58: 341-349.

13. George JN (1985) The role of membrane glycoproteins in platelet formation, circulation and senescence. Review and hypotheses. In: George JN, Nurden AT, Phillips DR (eds) Platelet Membrane Glycoproteins. Plenum Press, New York, pp 395-412.

14. Kelton JG, Steeves K (1983) The amount of platelet-bound albumin parallels the amount of IgG on washed platelets from patients with immune thrombocytopenia. Blood 62: 924-927.

15. Milton JG, Frojmovic MM (1979) Invaginated plasma membrane of human platelets: evagination and measurement in normal and 'giant' platelets. J Lab Clin Med 93: 162-170.

16. Frojmovic MM, Milton JG (1982) Human platelet size, shape and related functions in health and disease. Physiol Rev 62: 185-261.

17. Lurhuma AZ, Riccomi H, Masson PL (1977) The occurrence of circulating immune complexes and viral antigen in idiopathic thrombocytopenic purpura. Clin Exp Immunol 28: 49-55.

18. Trent RJ, Clancy RL, Danis V, Barten A (1980) Immune complexes in thrombocytopenic patients: cause or effect? Brit J Haematol 44: 645-654.

19. Helmerhorst FM, Smeenk RJT, Hack CE, Engelfriet CP, Borne AEG Kr von dem (1983) Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies. Brit J Haematol 55: 533-545.

20. Hedge UM, Williams K, Devereux S, Bowes A, Powell D, Fischer D (1983) Platelet-associated IgG and immune thrombocytopenia in lymphoproliferative and autoimmune disorders. Clin Lab Haematol 5: 9-15.

21. Puram V, Guiliani D, Morse BS (1984) Circulating immune complexes and platelet IgG in various diseases. Clin Exp Immunol 58: 672-676.

22. Moore A, Nachman RL (1981) Platelet Fc receptor. Increased expression in myeloproliferative disease. J Clin Invest 67: 1064-1071.

23. Gowland E, Kay HEM, Spillman JC, Williamson JR (1969) Agglutination of platelets by a serum factor in the presence of EDTA. J Clin Pathol 22: 460-462.

24. Shulman NR, Leisinger CA, Hotchkin AJ, Kant CA (1982) The nonspecific nature of platelet-associated IgG. In: Transactions of the Association of American Physicians, vol. XCV: 213-220.

25. Kelton JG, Carter CJ, Rodger C, Bebenek G, Gauldie J, Sheridan D, Kassam YB, Kean WF, Buchanan WW, Rooney PJ, Bianchi F, Denburg J (1984) The relationship among platelet-associated IgG, platelet lifespan and reticuloendothelial cell function. Blood 63: 1434-1438.

26. Leeuwen EF van, Ven JTM van der, Engelfriet CP, Borne AEG Kr von dem (1982) Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 59: 23-26.

27. Woods VL, Oh EH, Mason D, McMillan R (1984) Autoantibodies against the platelet glycoprotein IIb-IIIa complex in patients with chronic ITP. Blood 63: 368-375.

28. Woods VL, Kurata Y, Montgomery RR, Tani P, Mason D, Oh EH, McMillan R (1984) Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood 64: 156-160.

29. Mason D, McMillan R (1984) Platelet antigens in chronic idiopathic thrombocytopenic purpura. Brit J Haematol 56: 529-534.

30. Beardsly DS, Spiegel JE, Jacobs MM, Handin RI, Lux IV SE (1984) Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J Clin Invest 74: 1701-1707.

31. Ahn YS, Harrington WJ, Simon SR, Mylvaganam R, Pall LM, So AG (1983) Danazol for the treatment of idiopathic thrombocytopenic purpura. New Engl J Med 308: 1396-1399.

32. Imbach P, Barandun S, d'Apuzzo V, Baumgartner C, Hirt A, Morell A, Rossi E, Schöni M, Vest M, Wagner HP (1981) High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood. Lancet i: 1228-1231.

33. Fehr J, Hoffman V, Kappeler U (1982) Transient reversal of thrombocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous gammaglobulin. New Engl J Med 306: 1254-1258.

34. Newland AC, Treleaven JJ, Minchinton RM, Waters AH (1983) High-dose intravenous IgG in adults with auto-immune thrombocytopenia. Lancet i: 84-87.

35. Tsubakio T, Kurata Y, Katagiri S, Kankura Y, Tamaki T, Kuyama J, Kangama Y, Yonezawa T, Tarai S (1983) Alteration of T-cell subsets and immunoglobulin synthesis *in vitro* during high-dose gammaglobulin therapy in patients with idiopathic thrombocytopenic purpura. Clin Exp Immunol 53: 697-702.

36. Fridman WH, Néauport-Sautés C, Daëron M, Yodoi J, Löwy I, Brézin C, Vaquero C, Gelabert MJ, Thèze J (1984) Induction of Fc receptors and

immunoglobulin-binding factors in T-cell clones. Mol Immmunol 21: 1243-1251.

CHAPTER II

QUANTIFICATION OF PLATELET-BOUND ALLOANTIBODIES BY RADIOIMMUNOASSAY

A study on some variables

J.J.E. Vos, J.G. Huisman, I.N. Winkel, N.J. Risseeuw-Bogaert, C.P. Engelfriet and A.E.G. Kr. von dem Borne

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

SUMMARY

Several variables that may affect accurate measurement of platelet-associated IgG (PA-IgG) were studied by using a radioimmunoassay of the consumption type. The amount of PA-IgG of washed, unfixed normal donor platelets was 1.0 ± 0.9 fg IgG/platelet (m ± 2 sd). Upon storage of washed platelets in a buffer containing EDTA, the amount decreased significantly to 0.2 ± 0.2 fg IgG/platelet. Simultaneously, an increase in modal platelet volume was observed. Similar results were obtained when platelets were fixed with paraformaldehyde (PFA). We postulate that this decrease in PA-IgG is caused by the release of plasma IgG entrapped by the surface-connected canalicular system of the platelet when the platelets swell during storage in EDTA or fixation with PFA. A good quantification of platelet-bound alloantibodies was possible with our assay. This was demonstrated with different anti-Zw^a (= anti-$P1^{A1}$), anti-Bak^a and anti-HLA sera. We also observed that fragments of platelets as well as fragments of cells of other types can cause aspecifically increased PA-IgG values and can thus interfere with the proper measurement of platelet-bound antibodies in all kinds of immunoassays in general.

Submitted for publication

INTRODUCTION

In the past decade, many assays have been developed to measure platelet-associated immunoglobulin G (PA-IgG), presumed to be autoantibodies bound to the platelets of patients with idiopathic thrombocytopenia (ITP) (McMillan, 1981; von dem Borne, 1984). However, the nature of PA-IgG is not yet clear and the amounts of IgG measured on platelets of normal donors and of thrombocytopenic patients differ greatly. Moreover, the variables involved in PA-IgG measurement have mostly not been studied in detail and no attempt has been made to evaluate the methods by applying well-defined anti-platelet antibody-containing sera.

In this report, we describe the results obtained with a radioimmunoassay (RIA) for the measurement of PA-IgG, developed in our laboratory. Several variables that might influence PA-IgG measurement were studied. The ability of the test to quantify platelet-bound antibodies was determined by measurements on normal donor platelets sensitized with various sera containing platelet antibodies.

MATERIALS AND METHODS

Human IgG (HIgG)

HIgG, used for the calibration curve and the coating of the polystyrene beads, was derived from donor plasma by fractionation according to Cohn (fraction II). The HIgG had been treated at pH 4 with traces of pepsin to make it suitable for intravenous use (Immunoglobulin CLB) (Vos *et al.*, 1985). For construction of the calibration curve, stock solutions of 1 ml each containing 1 mg IgG (as determined by turbidometric analysis) were prepared and kept at -20°C. Each stock solution was frozen and thawed at the most four times.

Polystyrene beads

Polystyrene beads (Spherotech, diameter 6.35 mm) were coated in a 50-mM $NaHCO_3$ buffer (pH 9) containing human IgG (200 µl/bead) during a 16-h incubation under gentle rocking. Unbound IgG was removed by washing with phosphate-buffered saline (PBS), pH 7.4, containing 0.005% Tween-20 (v/v). The beads were rinsed twice in distilled water, freeze-dried and stored at 4°C until use. For optimal reactivity in the radioimmunoassay, the amount of human IgG attached to the polystyrene beads had to be as low as possible to minimize the risk of loss of coated IgG during incubation. Maximal binding of ^{125}I-anti-IgG to the bead (50% of input radioactivity) was reached at a minimum coating concentration of 2.5 µg human IgG/ml.

Radio-labelled anti-IgG

Affinity-purified rabbit-anti-human IgG (CLB no. M1023 KH16P) was treated with 20 mg pepsin/g IgG in acetic acid at pH 4.5 to yield $F(ab')_2$ fragments. The fragments were purified from undigested IgG by gel filtration on Sephacryl-200. The homogeneity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis, according to Laemmli (1970). The $F(ab')_2$ fragments were iodinated by the Iodogen method (Fraker & Speck, 1980) and subsequently freed from unbound ^{125}I by gel filtration over an ACA-44 column that had been equilibrated with a PBS-containing 0.2% bovine serum albumin (BSA). Peak fractions were pooled and stored at -20°C until use. The conjugate did not react with highly purified human IgM paraprotein. (See also Fig. 1.)

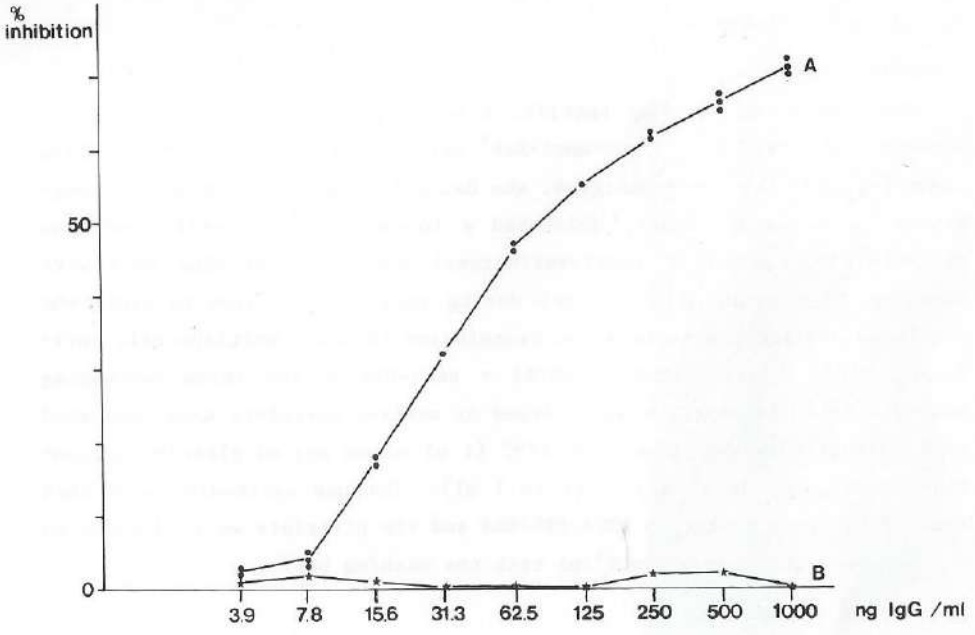

Fig. 1 - A. Standard curve obtained with human IgG (Cohn fraction II) and ^{125}I-labelled rabbit-anti-human IgG $F(ab')_2$. Accurate range between 7.8 and 62.5 ng IgG/ml. B. Standard curve with purified IgM.

Isolation of platelets

Platelet-rich plasma (PRP) was separated from blood collected in 10-ml tubes containing 4 mM Na_2EDTA (Venoject). Erythrocytes and leukocytes were pelleted by centrifugation during 10 min at 350 g. Platelets were pelleted from the PRP by centrifugation for 7 min at 2000 g and were washed 3 times in a solution of Na_2EDTA (9 mM) in PBS, pH 7.4, with 0.2% BSA (EDTA-PBS-BSA), resuspended in washing buffer and counted in a Coulter ZF automatic blood counter (diameter 50 microns, attenuation 0.500, aperture 4, threshold 10). Platelets were adjusted to a final concentration of about $5x10^7$/ml with washing buffer. Only three washings were applied, because the amounts of measured PA-IgG no longer diminished when platelets were washed more often.

Platelet fixation

Washed platelets were pelleted and resuspended in 1 ml of 1% (v/v) paraformaldehyde (PFA) in PBS for 5 min at room temperature, washed twice, resuspended in EDTA-PBS-BSA and adjusted to a concentration of about 5 x 10^7/ml in EDTA-PBS-BSA.

Platelet sensitization

Sera containing platelet-specific alloantibodies (anti-Zw^a = anti-Pl^{A1} sera: B., D., Pl., V., T. and anti-Bak^a sera: K and Pi) were obtained from women negative for these antigens, who had been alloimmunized during pregnancies and, as a result, delivered a thrombocytopenic child, or from patients, who developed posttransfusional purpura. Anti-HLA sera were obtained from women alloimmunized during pregnancy or from patients who developed adverse reactions after transfusion (CLB23 = multispecific anti-HLA-B; CLB32 = anti-HLA-A1; CLB184 = anti-HLA-A2 and serum containing anti-HLA-B7). In short, washed, fixed or unfixed platelets were incubated with alloantisera for 30 min at 37°C (1 ml serum per ml platelet suspension, containing $3x10^8$ platelets in 1 ml). Unbound antibodies were then removed by three washes in EDTA-PBS-BSA and the platelets were adjusted to a final concentration of $5x10^7$/ml with the washing buffer.

Sonication of platelets

Samples of 1 ml PRP were sonicated for 15 sec in an MSE sonicator, type 'Cabinet' no.7100, with a probe (tip diameter 3 mm) at an amplitude of 8 microns peak to peak. A 15-sec period revealed a population of disrupted

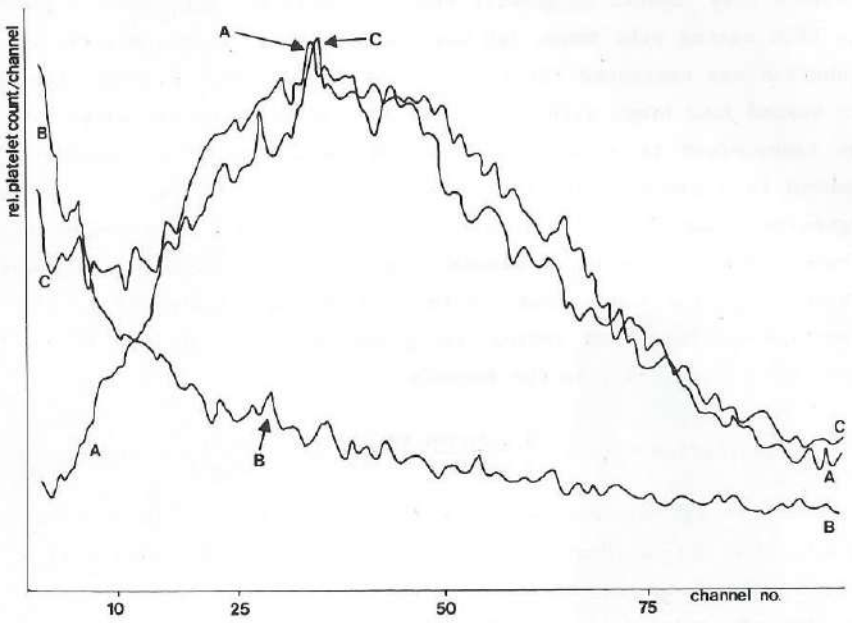

Fig. 2 - A) Size distribution plot of normal intact platelets. B) Size distribution plot of 15-sec-sonicated PRP. C) Size distribution plot of intact platelets after the addition of 15-sec-sonicated platelets, washed three times with EDTA-PBS-BSA.

platelets as observed by a Coulter Channalyzer (see also Fig. 2) and by microscopical examination.

Platelet immunofluorescence test (PIFT)

This test has been described in detail elsewhere (5). In brief, platelets were prepared, subsequently fixed, then sensitized with antiserum and washed three times. The platelets were then incubated with FITC-labelled rabbit-anti-human IgG (Dakopatts F202), appropriately diluted in PBS-0.2% BSA, washed twice, resuspended in a glycerol-PBS mixture and examined under the fluorescence microscope. Results were expressed as -, +, (+), 1+, 2+, 3+, 4+ (scored arbitrarily as 0, 0.25, 0.5, 1, 2, 3, 4).

Radioimmunoassay for PA-IgG

Platelet suspension (100 µl) was incubated with 100 µl of ^{125}I-radiola-

belled $F(ab')_2$ fragments of rabbit-anti-human IgG for 16 h at 4°C in a polystyrene tray (Abbott Diagnostic Products, Chicago, ILL, USA). A polystyrene bead coated with human IgG was added to the reaction mixture and the incubation was continued for 6 h at room temperature. Finally, the beads were washed four times with distilled water in an automatic washing device, then transferred to a counting tube and the amount of radioactivity was measured in a gamma scintillation counter. Five dilutions of a platelet suspension (range 50 - 25 - 12.5 - 6.25 - 3.13×10^6/ml) were tested in triplicate. Simultaneously, a standard IgG reference curve was prepared in triplicate with a range from 7.8 to 62.5 ng human IgG/ml (Fig. 1). The percentage inhibition of radioactivity (RA) bound to the bead by the test sample was calculated from the formula:

$$\% \text{ inhibition} = \left(1 - \frac{\% \text{ unbound RA by test sample}}{\% \text{ unbound RA by blank sample}}\right) \times 100\%$$

The amount of IgG corresponding with this percentage inhibition was read directly from the calibration curve and was subsequently correlated to the number of platelets present in the test sample. In general, three or four dilutions of a platelet suspension appeared to have IgG values within the range of the calibration curve. From these measurements the average PA-IgG value was calculated and expressed in fg IgG/platelet. Binding of ^{125}I-anti-IgG was maximally 50% of the added radioactivity. Of this amount, maximally 70% could be inhibited by the addition of human IgG.

RESULTS

Absorption of the platelets to the IgG-coated bead

Absorption of platelets in the test sample to the IgG-coated beads could lead to false low values of the inhibition percentage. That this occurred was excluded as follows. Platelets, unsensitized or sensitized with anti-Zw^a, were incubated with ^{125}I-labelled anti-IgG and subsequently washed. An IgG-coated bead was added and the incubation was continued. After 6 h, the beads were washed and the amount of bound radioactivity was measured. Binding by unsensitized platelets was maximally 1.2% of the total amount of radioactivity bound to the platelets, whereas binding of sensitized platelets to the bead was maximally 2.5%.

In another series of experiments, platelets were removed from the incubation mixture before the human IgG-coated bead was added. Similar PA-IgG values were observed as when platelets were still present during incubation with the bead. From these experiments, absorption of platelets to the beads appeared to be of minor proportions and not to affect the test results. Therefore, removal of platelets after the first incubation step was not necessary.

Affinity of radio-labelled anti-IgG for platelet-associated versus free human IgG

Rosse *et al.* (1984) have suggested that the binding affinity of the radio-labelled anti-IgG is different for IgG associated to platelets as compared with IgG in solution (free IgG) used for the calibration curve. This could give rise to falsely elevated PA-IgG values. To investigate this, a series of twofold dilutions of a platelet suspension was prepared to which a calibrated amount of human IgG was added. The amount of radio-labelled anti-IgG bound by the platelets, corrected for the added amount of 'free' IgG, was identical with the amount bound by the platelets in a suspension without added IgG (Table I).

TABLE I

The affinity of the radio-labelled anti-IgG for PA-IgG and/or 'free' IgG

Platelet count ($\times 10^7$/ml in the test sample	Platelet-associated IgG measured (ng/ml)		Difference (ng/ml)
	with free IgG	without free IgG	
5	37	25	12.0
2.5	26.4	12.4	13.6
1.25	20	6.1	13.9

Washed platelets were tested in three concentrations, with and without IgG (13.5 ng/ml) added to the suspensions. The RIA for PA-IgG was performed as described in Materials and Methods.

Storage of platelets

In our laboratory, blood samples from the patients under investigation are mostly tested one day, and sometimes 2 days, after collection. Therefore, we investigated the effect of storage of platelets in whole blood on the PA-IgG values as measured. In 10 normal donors as well as in 10 patients, PA-IgG values of platelets from freshly collected EDTA blood were

identical with those of platelets from blood kept for one or two days at 4°C. Also platelet number and modal platelet size (as determined on the Coulter Counter Channelyzer) did not change. These results are in accord with those of others (Hedge *et al*, 1984).

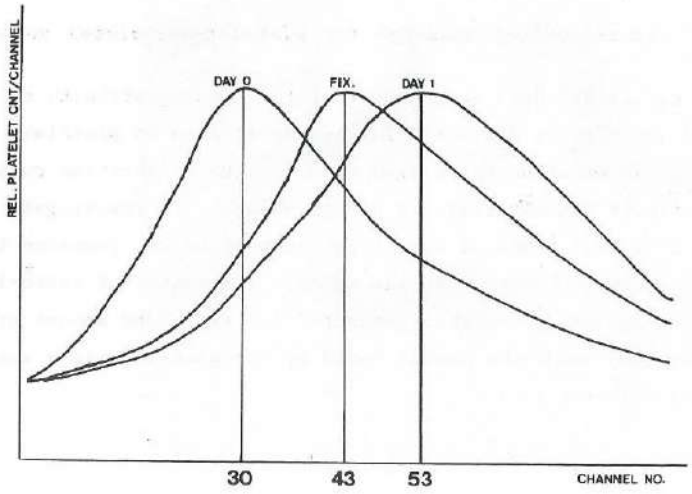

Fig. 3 - Modal platelet size determined by the Coulter Channelyzer (schematically drawn). a) Freshly prepared unfixed platelets: (m ± sd) channel 30 ± 4, n = 38. b) Freshly prepared PFA-fixed platelets: channel 43 ± 5, n = 20. c) Unfixed platelets stored at 4°C for one day: channel 53 ± 18, n = 38. All differences were significant ($p<0.001$, Wilcoxon-matched pairs signed ranks test).

Storage of EDTA-washed platelets

We also studied the effect of storage on suspensions of platelets in EDTA-PBS-BSA. The platelet suspensions were stored at 4°C for either one or two days. The platelets were then washed again once in EDTA-PBS-BSA before the determination of PA-IgG. PA-IgG levels measured on one-day-old EDTA-washed platelets appeared to be significantly lower when compared with those of platelets prepared and analysed on the same day (day 0: 1.0± 0.9 fg/platelet; day 1: 0.2 ± 0.2 fg/platelet, m ± 2 sd, Wilcoxon-matched pairs signed ranks test $p<<0.001$, n=38). The modal platelet size, as determined in the Coulter Counter Channelyzer (expressed as channel number),

increased simultaneously (m ± 2 s.d. day 0, 30 ± 4, day 1, 53 ± 18, Wilcoxon-matched pairs signed ranks test $p<<0.001$, n = 38). (See also Fig. 3.) Two-day-stored EDTA-washed platelets appeared to have equal amounts of PA-IgG as did one-day-stored platelets. Moreover, no further increase of modal platelet size was observed.

To exclude the possibility that the decrease in PA-IgG was a result of a shift in the equilibrium of platelet-bound IgG to 'free' IgG in the EDTA-PBS-BSA buffer, we stored platelet suspensions in the presence of human IgG (Cohn fraction II) at 1 mg/ml. A decrease in the PA-IgG observed after storage in EDTA-PBS-BSA was again found, indicating that the decrease is not the result of an equilibrium shift of platelet-bound IgG (m ± 2 sd day 1 without IgG 0.3 ± 0.2, day 1 with IgG 0.3 ± 0.2 fg/platelet, $p > 0.1$, Wilcoxon-matched pairs signed ranks test, n = 5). In addition, this experiment showed that the addition of external IgG (1 mg/ml or less) did not result in increased PA-IgG values.

Platelet fixation

Because platelets are routinely fixed with PFA for the PIFT, the effect of PFA fixation on PA-IgG values in normal donors was studied. PFA fixation led to significantly decreased PA-IgG levels in normal donors (m ± 2 sd 0.3 ± 0.3 fg/platelet, Wilcoxon-matched pairs signed ranks test $p<0.001$, n = 20). A concomitant rise of modal platelet size was observed (m ± 2 sd 43 ± 5; Wilcoxon-matched pairs signed ranks test $p<0.001$ n=20). The amount of PA-IgG on PFA-fixed platelets did not alter when they were subsequently stored for one or two days at 4°C in EDTA-PBS-BSA.

Effect of sonication on PA-IgG

Platelets are known to contain intracellular IgG (Kelton *et al*, 1980; Dixon *et al*, 1983), which is released when sonication or lysis-inducing agents are applied. The amount of IgG freed by sonication on the day of platelet preparation was compared with the amount of IgG freed by sonication of platelets from the same donor stored for one day in EDTA-PBS-BSA at 4°C. On both days, about the same amount of IgG (1.5-2.0 fg HIgG/platelet) was released from the platelets.

Quantification of amount of specifically bound PA-IgG

Platelets were sensitized with seven sera containing platelet-specific alloantibodies (anti-Zw^a = anti-Pl^{A1} and anti-Bak^a) and four sera containing HLA antibodies (directed against HLA-A1,-A2, -B7 and multiple HLA-B

antigens, respectively) to determine whether specifically bound antibodies could be measured in our assay. Simultaneously, the degree of sensitization of the platelets was determined in the PIFT. As shown in Table II, it appeared that donor platelets positive for each of these antigens showed an elevated amount of PA-IgG upon sensitization, whereas donor platelets negative for these antigens did not. Incubation with sera from normal healthy AB donors did not result in elevated PA-IgG values.

TABLE II

Results of the sensitization of normal donor platelets with various alloantisera

Phenotype of the donor	x anti-Zw(a)	x anti-Bak(a)	anti-HLA -A1	-A2	-B7	-pan B
1. Zw(a+)Bak(a+)	14.5	2.4				
2. Zw(a+)Bak(a+)	4.3	5.6				
3. Zw(a+)Bak(a-), HLA (B7+)	4.8	0.3			2.0	
4. Zw(a+)Bak(a-), HLA (B7-)	1.6	0.3			0.3	
5. Zw(a-)Bak(a+), HLA (A1,A2,B51+)	0.2	2.3	3.2	3.1		12.8
6. Zw(a-)Bak(a+), HLA (A2-)	0.1	1.7		0.5		
7. HLA (A2+)				2.7		
8. Zw(a+)Bak(a+)	14.1	1.0				

The platelets from 8 normal healthy donors were sensitized with anti-Zw^a B, anti-Bak^a K, anti-HLA-A1, -A2, -B7 and multispecific anti-HLA-B as described in Materials and Methods. All donor platelets had a PA-IgG lower than 0.8 fg/platelet after incubation with normal AB serum. In donor 4, sera had to be diluted with AB substance (1:2) because the blood group was AB. In the other experiments, there was no ABO antagonism between cells and sera.

The effects of storage and of fixation of sensitized platelets in EDTA-PBS-BSA on the amount of PA-IgG was also studied. Upon storage in EDTA-PBS-BSA for 1 day at 4°C, anti-Zw^a-sensitized platelets lost some PA-IgG (28%, m ± sem; day 0, 6.4 ± 1.6 fg/platelet; day 1, 4.6 ± 1.2 fg/platelet, n=7). Fixation after platelet sensitization led to a decrease of 24% of PA-IgG as compared with that for unfixed sensitized platelets (m ± sem, 4.9 ± 2.0 fg/platelet, n=7).

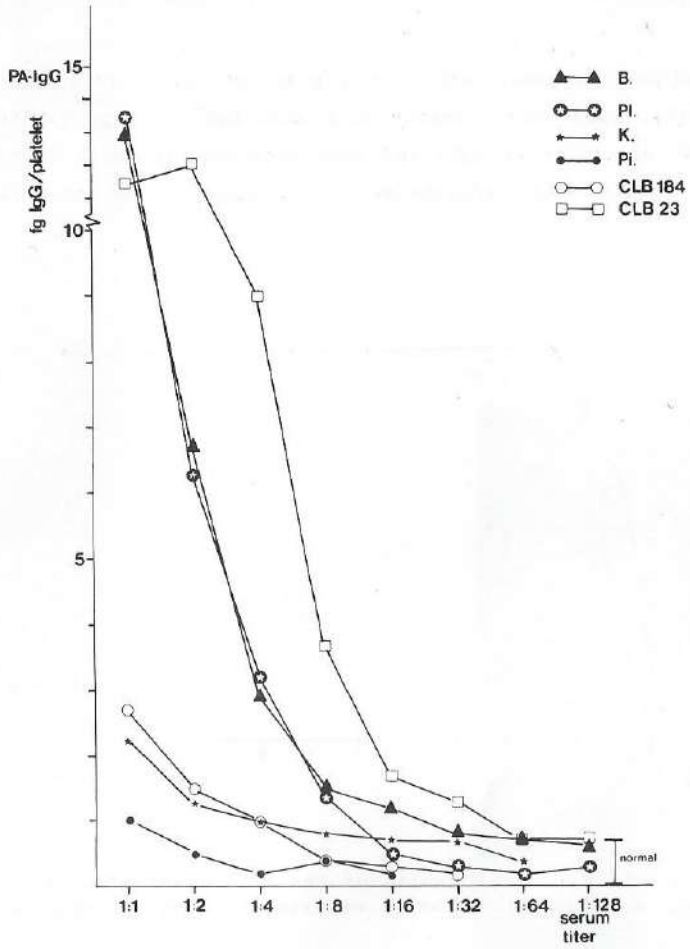

Fig. 4 - Titration curves of platelet-specific alloantisera (anti-Zw^a, B and Pl, anti-Bak^a, K and Pi) and HLA antisera (CLB 184 = anti-HLA-A2 and CLB 23 = multispecific anti-HLA-B), incubated with the platelets of donors positive for these antigens. The antisera were diluted in AB serum.

Because, in the indirect PIFT, platelets are routinely fixed before sensitization, it was of interest to test whether the amount of PA-IgG on platelets fixed before sensitization would be different from PA-IgG measured on platelets fixed after sensitization. The results obtained after sensitization with anti-Zw^a before and after fixation of the platelets differed significantly (m ± sem, before fixation 8.5±1.5 fg/platelet, after fixation 4.1±0.7 fg/platelet, n=5, Wilcoxon-matched pairs signed ranks test $p<0.001$). The highest PA-IgG values, measured upon sensitization with

anti-Zw^a and postfixation, were 14.5 fg/platelet (i.e., about 54 000 molecules of IgG), upon sensitization with anti-Bak^a 5.6 fg/platelet (i.e., about 21 000 molecules of IgG) and upon sensitization with multispecific anti-HLA-B (CLB23) 12.8 fg/platelet (i.e., about 48 000 molecules IgG).

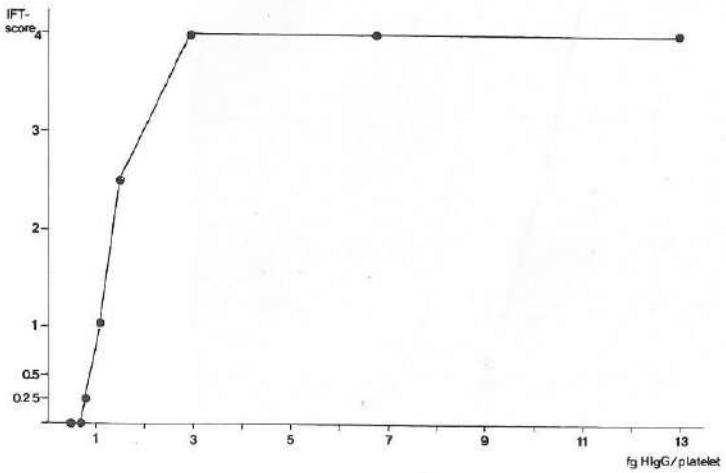

Fig. 5 - Comparison of the results of the PIFT (with FITC anti-IgG) and PA-IgG values measured in titration experiments with anti-Zw^a serum (B).

Series of dilutions of the various alloantibody-containing sera were prepared (1:2 - 1:64/128) to determine the sensitivity of our radioimmunoassay. Fig. 4 shows the titration curves of four platelet-specific antisera and two anti-HLA antisera. Similar results were obtained with other anti-Zw^a sera. The results were compared with the results obtained in the simultaneously performed (semi-quantitative) PIFT. The lowest detection range of the two tests appeared to be about the same (Fig. 5), but the range in which PIFT could discriminate between various grades of sensitization was rather small. Any quantity above 3.0 fg IgG/platelet was judged as 4+ in the PIFT (i.e. maximal sensitization), whereas the PA-IgG assay could still differentiate between quantities of antibodies bound above 3.0 fg IgG/platelet (Fig. 5).

Interference of platelet fragments with proper PA-IgG measurement

The presence of fragmented platelets or other (IgG-coated or uncoated) cell particles in the test suspension might interfere with the exact measurement of PA-IgG (Shulman et al, 1982). These fragments must be pelletted together with the platelets during platelet preparation to be able to interfere. Therefore, PRP containing unsensitized platelets or platelets sensitized with anti-Zw^a was sonicated and added to non-sonicated PRP (ratio 2:1). Subsequently, the mixture was washed, fixed and counted. The presence of platelet fragments was confirmed by the Coulter Channelyzer (see Fig. 2) and by flowcytometry. PA-IgG levels were then determined and compared with PA-IgG levels of the intact cells without added fragments.

TABLE III

PA-IgG measurement of sensitized and unsensitized platelets of a Zw(a+) donor, with or without the addition of fragmented platelets or erythrocytes before platelet isolation

	No fragments added	Sonicated platelets added unsensitized	Sonicated platelets added sensitized with anti-Zw^a	Sonicated erythrocytes added
Unsensitized platelets	0.3*	1.1	1.4	0.8
Anti-Zw^a-sensitized platelets	3.4	3.6	4.5	5.2

* fg IgG/platelet.

As shown in Table III, PA-IgG measured in a suspension with platelet fragments was more elevated than PA-IgG measured in a suspension containing only intact platelets, and higher amounts were found with fragments from sensitized platelets than with fragments from unsensitized platelets. Also, elevated amounts of PA-IgG were observed when fragmented erythrocytes were added.

DISCUSSION

In the many publications on the measurement of platelet-associated IgG (PA-IgG), the reported values in normal donors (0.1-26 fg/platelet) and in patients with ITP (0.1 - 1800 fg/platelet) vary markedly (McMillan, 1981; von dem Borne, 1984). In most assays, PA-IgG values in normal donors are

10 to 20 times higher than expected when the amount of PA-IgG is determined only by the number of Fcγ-receptor molecules present on the platelet (0.1-0.5 fg/platelet) (Karas *et al*, 1982). Only LoBuglio *et al* (1983) reported a radioimmunoassay by which such amounts were detected on the platelets of normal individuals. We observed PA-IgG values similar to those reported by LoBuglio *et al*, when platelets were stored for 16 h at 4°C in EDTA-PBS-BSA or when platelets were fixed with PFA. In the assay of LoBuglio *et al*, density-gradient centrifugation and multiple extra washings are necessary which may result in the loss of platelets. These stages are not necessary in our assay, which is also more easily performed, because of the handiness of the polystyrene beads, the incubation trays and the automatic washing device.

Rosse *et al* (1984) have argued that, in direct assay systems, one would measure lower PA-IgG levels than in assays of the consumption type, such as ours. The affinity of anti-IgG for platelet-bound IgG would be different from that of 'free' IgG, which is usually used as reference standard for consumption assays. However, we did not find any differences in the amount of PA-IgG of normal donor platelets in the absence or the presence of added soluble IgG and we, therefore, conclude that this presumed difference in affinity probably does not play a role in our assay system.

Our results show that the amount of measured PA-IgG in normal donors upon storage in EDTA-PBS decreased from 1.0 ± 0.9 fg/platelet to 0.2 ± 0.2 fg/platelet. This decrease during storage was not caused by simple elution of IgG, because storage of the platelets in a medium containing soluble IgG led to a similar decrease. A concomitant increase of the modal platelet size was seen, and similar results were obtained when the platelets were fixed with PFA. From this, we postulate that the decrease in PA-IgG upon storage in EDTA-PBS or PFA fixation is caused by platelet swelling and the release of plasma IgG entrapped in the platelet surface-connected canalicular system (SCCS) (Kelton & Steeves, 1983). Upon swelling, the SCCS is enfolded and incorporated into the platelet surface (Milton & Frojmovic, 1979; Frojmovic & Milton, 1982). When platelets are tested immediately after preparation and without fixation, the IgG in the SCCS is expelled during the overnight incubation, leading to increased PA-IgG values. The effect of EDTA seems to be time- and/or dose-dependent as swelling does not occur upon storage in whole EDTA blood (containing 4 mM EDTA) and becomes maximal after storage during 16 h at 4°C. Apparently,

PFA fixation induces swelling much faster (in a few minutes). PFA fixation could additionally affect PA-IgG by blockage of the platelet Fcγ receptor (Helmerhorst *et al*, 1983).

Sensitization of platelets with different antisera and dilutions thereof (anti-Zw^a, anti-Bak^a, anti-HLA-A1, -A2, -B7 and multispecific anti-HLA-B) confirmed the ability of our assay to quantify platelet-bound antibodies with high sensitivity. Moreover, sensitized platelets did show a moderate decrease of PA-IgG upon storage or PFA fixation (28 and 24%, respectively) too, which was somewhat higher than found with unsensitized platelets. It is not clear whether this was caused by the loss of aspecifically bound or entrapped IgG or by the elution of some specifically bound platelet antibody IgG.

When platelets were fixed with PFA after platelet sensitization with alloantibodies, significantly higher PA-IgG values were found than with platelet fixation before sensitization. Previously, this difference was not observed in the PIFT (Helmerhorst *et al*, 1980), but this may have been because, as we have found in the present study, the quantitative detection range of the PIFT is much smaller than that of the RIA. The difference found between pre- and postfixation may be caused by some loss of antigenic sites during prefixation.

When fixation was applied after sensitization with anti-Zw^a serum, we found that, with anti-Zw^a antisera, maximally 14.5 fg of IgG was bound to the platelets of a Zw^a-positive donor, i.e. about 54 000 molecules of IgG. Although in our binding studies no saturation of the antigens appeared to be obtained, these amounts of IgG measured by our PA-IgG assay correspond with the number of glycoprotein IIb-IIIa molecules (about 40-50 000) on the platelet membrane (McEver *et al*, 1980), which are the sites of the Zw^a antigens (Kunicki & Aster, 1979).

Apart from plasma IgG entrapped in platelet surface-connected cannaliculi, other factors may contribute to false elevated PA-IgG levels in patients with thrombocytopenia. These are the following.

1. PA-IgG measurements are often performed on solubilized or sonicated platelets. Various amounts of intracellular IgG are liberated from the platelets by these procedures (Kelton *et al*, 1980; Dixon *et al*, 1983; this study).

2. Evidence has been produced for the occurrence of platelet fragments in the blood of thrombocytopenic patients (Khan *et al*, 1975; Zucker-Frank-

lin & Karpatkin, 1977; Shulman *et al*, 1982). Because these fragments are not (accurately) counted, but contribute to the amount of IgG in the test suspension, their presence could lead to false elevated PA-IgG values, as we found.

3. Antibodies against platelet cryptantigens, which react only in the presence of EDTA, possibly without any pathological significance (Pegels *et al*, 1982; von dem Borne *et al*, 1985), may cause elevated PA-IgG values when measurements are done on platelets from EDTA blood, washed and suspended in EDTA-containing solutions.

4. Circulating immune complexes may interfere in PA-IgG measurement (Helmerhorst *et al*, 1983; Lurhuma *et al*, 1977; Trent *et al*, 1980; Hegde *et al*, 1983; Puram *et al*, 1984), although this risk is minimized by PFA fixation of the platelets (Helmerhorst *et al*, 1983).

In conclusion, we have developed a radioimmunoassay of the consumption type, adapted in such a way that we could measure platelet-bound antibody IgG in a reliable and sensitive way. However, reliable measurement of platelet-bound autoantibody IgG in patients with autoimmune thrombocytopenia is still difficult, even with our assay. This is due to the presence of platelet fragments in the patient's blood which might interfere as well as to the occurrence of antibodies against platelet cryptantigens and immune complexes that bind to the patient's platelets *in vivo* or *in vitro*.

ACKNOWLEDGEMENTS

We thank Mrs. C.M. van der Plas-van Dalen for the supply of antisera and Mrs. W. Winkel-Groeneveld, Ms S. Scholten and Mrs. J. Gerritsen for their secretarial assistance. W. Schaasberg is acknowledged for his expert assistance in statistical analysis of the results.

REFERENCES

BORNE, A.E.G. Kr. von dem, VERHEUGT, F.W.A., OOSTERHOF, F., RIESZ, E. von, BRUTEL de la RIVIERE, A. & ENGELFRIET, C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. *British Journal of Haematology*, 39, 195-207.

BORNE, A.E.G. Kr. von dem (1984) Autoimmune thrombocytopenia. In: Engelfriet, C.P., Loghem, J.J. van and Borne A.E.G. Kr. von dem (eds.) *Immunohaematology*, Elsevier, Amsterdam, pp 222-256.

BORNE, A.E.G. Kr. von dem, LELIE, J. van der, VOS, J.J.E., PLAS, C.M. van der, RISSEEUW-BOGAERT, N.J., TICHELER, M.D.A. & PEGELS, J.G. (1986) Antibodies against cryptantigens of platelets. Characterization and significance for the serologist. In: Décary, F. and Rock, G.A. (eds.) *Platelet Serology, Research Progress and Clinical Implications*. Karger, Basel, pp 33-46.

DIXON, D.B., WENCEL-DRAKE, J.D. & GINSBERG, M.H. (1983) Localization of platelet-associated IgG in alpha granules. *Blood* 62, no. 5, suppl. page 243a.

FRAKER, P.J. & SPECK, J.C. (1980) Protein and cell membrane iodinations with sparingly soluble chloramide-1,2,4,6-tetrachloro-3α,6α-diphenyl glycoluril. *Biochem. Biophys. Res. Commun.* 4, 849-857.

FROJMOVIC, M.M. & MILTON, J.G. (1982) Human platelet size, shape and related functions in health and disease. *Physiological Reviews*, 62, 185-261.

HEGDE, U.M., WILLIAMS, K., DEVEREUX, S., BOWES, A., POWELL, D. & FISCHER, D. (1983) Platelet-associated IgG and immune thrombocytopenia in lymphoproliferative and autoimmune disorders. *Clinical and Laboratory Haematology*, 5, 9-15.

HEGDE, U.M., BOWES, A. & ROSER, B.L.T. (1984) The effect of storage in different anticoagulants on platelet-associated IgG. *Clinical and Laboratory Haematology*, 6, 51-59.

HELMERHORST, F.M., BOSSER, B., BRUIN, H.G. de, ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1980) Detection of platelet antibodies: a comparison of three techniques. *Vox Sanguinis*, 39, 83-92.

HELMERHORST, F.M., SMEENK, R.J.T., HACK, C.E., ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1983) Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies. *British Journal of Haematology*, 55, 533-545.

KARAS, S.P., ROSSE, W.F. & KURLANDER, R.J. (1982) Characterization of the IgG-Fc receptor on human platelets. *Blood*, 60, 1277-1282.

KELTON, J.G., DENOMME, G. & GAULDIE, J. (1980) The majority of platelet-associated IgG (PA-IgG is in the interior of platelets and not measurable using standard assays. *Transfusion*, 20I, 625-626.

KELTON, J.G. & STEEVES, K. (1983) The amount of platelet-bound albumin parallels the amount of IgG on washed platelets from patients with immune thrombocytopenia. *Blood*, 62, 924-927.

KHAN, I., ZUCKER-FRANKLIN, D. & KARPATKIN, S. (1975) Microthrombosis and platelet fragmentation associated with idiopathic autoimmune thrombocytopenia. *British Journal of Haematology*, 31, 449-460.

KUNICKI, T.J. & ASTER, R.H. (1979) Isolation and immunologic characterization of the human platelet allo-antigen Pl^{A1}. *Molecular Immunology*, 16, 353-360.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

LoBUGLIO, A.F., COUNT, W.S., VINOCUR, L., MAGLOTT, G. & SHAW, G.M. (1983) Immune thrombocytopenic purpura. Use of a ^{125}I-labelled anti-human IgG monoclonal antibody to quantify platelet-bound IgG. *New England Journal of Medicine*, 309, 459-463.

LURHUMA, A.Z., RICCOMI, H. & MASSON, P.L. (1977) The occurrence of circulating immune complexes and usual antigen in idiopathic thrombocytopenic purpura. *Clinical and Experimental Immunology*, 28, 49-55.

McEVER, R.P., BAENZIGER, N.L. & MAJERUS, P.W. (1980) Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. *Journal of Clinical Investigation*, 66, 1311-1318.

McMILLAN, R. (1981) Chronic idiopathic thrombocytopenic purpura. *New England Journal of Medicine*, 304, 1135-1147.

MILTON, J.G. & FROJMOVIC, M.M. (1979) Invaginated plasma membrane of human platelets: evagination and measurement in normal and 'giant' platelets. *Journal of Laboratory and Clinical Medicine*, 93, 162-170.

PEGELS, J.G., BORNE, A.E.G. Kr. von dem, BRUYNES, E.C.E. & ENGELFRIET, C.P. (1982) Pseudothrombocytopenia: an immunological study on platelet antibodies dependent on ethylene diamine tetra-acetate. *Blood*, 59, 157-161.

PURAM, V., GIULIANI, D. & MORSE, B.S. (1984) Circulating immune complexes and platelet IgG in various diseases. *Clinical and Experimental Immunology*, 58, 672-676.

ROSSE, W.F., DEVINE, D.V. & WARE, R. (1984) Reactions of immunoglobulin G-binding ligands with platelets and platelet-associated immunoglobulin G. *Journal of Clinical Investigation*, 73, 489-496.

SHULMAN, N.R., LEISINGER, C.A., HOTCHKIN, A.J. & KANT, C.A. (1982) The nonspecific nature of platelet-associated IgG. In: *Transactions of the Association of American Physicians*, vol. XCV, pp 213-220.

TRENT, R.J., CLANCY, R.L., DANIS, V. & BASTEN, A. (1980) Immune complexes in thrombocytopenic patients: cause or effect? *British Journal of Haematology*, 44, 645-654.

VOS, J.J.E., LEEUWEN, E.F. Van, AKEN, W.G. Van, ENGELFRIET, C.P. & BORNE, A.E.G. Kr. Von dem (1985) Intravenous gammaglobulin therapy in idiopathic thrombocytopenic purpura. Results with the Netherlands Red Cross immunoglobulin preparation. *Vox Sanguinis*, 49, 92-100.

ZUCKER-FRANKLIN, D. & KARPATKIN, S. (1977) Red cell and platelet fragmentation in idiopathic autoimmune thrombocytopenic purpura. *New England Journal of Medicine*, 60, 834-840.

CHAPTER III

CLINICAL SIGNIFICANCE OF A POSITIVE PLATELET IMMUNOFLUORESCENCE TEST IN THROMBOCYTOPENIA

A.E.G. Kr. von dem Borne[1,2], J.J.E. Vos[1], J. van der Lelie[2], B. Bossers[1], and C.M. van der Plas-van Dalen[1]

[1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam;
[2]Department of Haematology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.

SUMMARY

The sensitivity and specificity of platelet immunofluorescence for the diagnosis of idiopathic thrombocytopenia (ITP) was studied in a consecutive series of 255 patients. Patients' platelets were tested directly. Ether eluates of these platelets and patients' sera were tested indirectly with normal donor platelets. Positive results were obtained for 92% of the ITP patients with a platelet count of $<150 \times 10^9$/l and even for 98.4% of the patients with a count of $<100 \times 10^9$/l. However, for many cases immunofluorescence scores were low: 59.8% of the patients had scores of ½-1. Most patients (93%) with a positive direct test had a positive indirect eluate test. Thus, in most, if not all patients, platelet-bound antibodies were present, but not platelet-bound immune complexes. Positive immunofluorescence tests were obtained for many patients with a diagnosis other than ITP which caused a low specificity of the test for this diagnosis. The low specificity was also due to the fact that frequently antibodies against platelet cryptantigens (EDTA- and paraformaldehyde-dependent antibodies) were present in the blood of many patients.

Submitted for publication

INTRODUCTION

Many tests have been developed to detect platelet auto-sensitization in patients with idiopathic thrombocytopenia (ITP) (von dem Borne, 1984). Most of these tests are based on the measurement of consumption of antiglobulin sera by the patient's platelets.

In general, the sensitivity of such assays for the detection of increased amounts of platelet-associated immunoglobulins in clinically diagnosed ITP is high. However, the diagnostic specificity of the consumption assays as well as of direct assays, such as the platelet radioactive antiglobulin test, has been found to be low (Mueller-Eckhardt *et al*, 1980; Kelton *et al*, 1982). This could be due to the occurrence of immune mechanisms in many more thrombocytopenic disorders than has previously been thought likely (Kelton *et al*, 1982). Another possibility is that non-immune mechanisms are responsible for increased amounts of platelet-associated immunoglobulins in certain diseases (Mueller-Eckhardt *et al*, 1980, 1982). Indeed Shulman *et al* (1982) have suggested that such a mechanism might be platelet fragmentation, known to occur in many thrombocytopenic disorders, including ITP. Platelet fragments are not counted as platelets, but they may contribute to the amount of platelet-associated immunoglobulins measured, either because they are also sensitized by autoantibodies or because they may bind plasma proteins non-specifically.

For many years, we have applied the platelet immunofluorescence test (PIFT) to detect platelet auto-sensitization in ITP (von dem Borne *et al*, 1980). The advantage of this test is that binding of immunoglobulin and complement to intact platelets is directly visualized, without interference of platelet fragments. A disadvantage of the PIFT is that it is a semi-quantitative test in which only the fluorescence strength is scored, although this can be done quite reliably (Helmerhorst *et al*, 1980). Because of the essentially different approach used in this assay, it seemed worthwhile to study the diagnostic sensitivity and specificity of the PIFT in a consecutive series of patients, as has been also done for the platelet-associated IgG (PAIgG) assay (Kelton, 1982). The results of this study are presented here.

METHODS

The test has been described in detail before (von dem Borne *et al*, 1978, 1980). Direct immunofluorescence was performed on paraformaldehyde-fixed platelets from the patients. Ether eluates were prepared from unfixed patient's platelets. Eluates and sera from the patients were tested by indirect immunofluorescence on fixed normal donor platelets. As antiglobulin reagents, FITC anti-Ig, anti-IgG, anti-IgM, anti-IgA and anti-C3b were used.

In the standard tests, platelets from EDTA blood of both patients and normal donors were applied, suspended in phosphate-buffered saline (PBS) containing EDTA. However, when enough material was available, the eluates and sera were also tested with fixed platelets from citrate-anticoagulated donor blood, washed with and suspended in PBS. Moreover, unfixed platelets from EDTA blood were applied as well. The PIFT results were scored as follows: ++++=4, +++=3, ++=2, +=1, (+)=½ (weak), ±=¼ (dubious), 0=0 (negative).

The relation between the fluorescence score and the amount of antibody bound to platelets, as measured in a ^{125}I-anti-IgG consumption assay (Vos *et al*, 1986), is shown in Chapter II, Fig. 5.

In each patient, the platelet count of the EDTA blood sample was determined either by a Coulter S plus II counter or manually when fewer than 20×10^9 platelets per litre were counted or when the machine did not determine the platelet count because of a non-semilogarithmic platelet-size distribution.

PATIENTS

All patients whose blood was sent to our laboratory for platelet antibody testing in a period of 3 months were evaluated. We tested the blood of 233 patients at least once and of 22 patients repeatedly (2-4 times). Altogether, 278 tests were done. Clinical information was obtained from all patients via a questionnaire and/or by inquiry over the telephone. Because of insufficient amounts of platelets, the direct test failed in 4 patients and no eluates could be prepared for 27 patients. The indirect serum test was done for all patients except one, from whom no clotted blood was obtained.

The criteria for the diagnosis of ITP were the existence of an acquired thrombocytopenia without another disease, a normal bone marrow and a normal coagulation status.

Immunofluorescence studies on the blood of patients with idiopathic thrombocytopenia (ITP)

Of the 255 patients studied, 194 had thrombocytopenia (platelet count $<150x10^9/l$) and 75 of those were diagnosed as suffering from ITP. The results of the immunofluorescence test (IFT) results in these patients are shown in table I.

TABLE I

Immunofluorescence test results in 75 ITP patients with a platelet count of $<150x10^9/l$

Direct IFT	Number	%	Indirect IFT on eluate nr. positive/ nr. tested	Indirect IFT on eluate % positive	Indirect IFT on serum nr. positive/ nr. tested	Indirect IFT on serum % positive
Positive (score ½-4)	63	84.0	48/51	94.1	41/62	66.1
Dubious (score ¼)	4	5.3	3/4	75.0	1/4	25.0
Negative (score 0)	5	6.6	0/5	0	0/5	0
Not tested	3	4.0	0/0	-	3/3	100
Total	75	100	51/61	83.6	45/75	60.0

A positive direct IFT was obtained in 63 (84.0%) of the ITP patients, usually with a positive indirect IFT on the platelet eluate (94.1%) and often with the serum (66.1%). A dubious direct test result was found for 4 patients (5.3%). Of these, 3 were considered as positive, because the eluate test was also (weakly) positive (together with a positive serum test in 1). For 5 patients (6.6%), all three tests were negative. For 3 further patients, no platelets were isolated from the blood for direct testing and the preparation of an eluate. But the indirect serum test was positive in all three. From a second blood sample obtained later, enough platelets were obtained and both a positive direct test and an eluate test were found. Therefore, these 3 patients were also considered as being positive. Thus, negative immunofluorescence results were observed for only 6 of the 75 ITP patients and the sensitivity for the diagnosis of ITP was therefore 92.0%. This percentage was even higher when only patients with a more severe thrombocytopenia (platelet count $<100x10^9/l$) were considered, i.e. positive results in 62 of 63 patients or 98.4%.

The relationship between the score of the direct test and the platelet count for all ITP patients (including 15 who were retested and 11 in remission) is shown in Fig. 1. There was a marked variation in the score, even in patients with low platelet counts. Low scores (½-1) were much more frequently (59.8%) encountered than higher scores (2-4) (26.5%). But higher scores were more often associated with lower platelet counts and there was a significant relationship between these two parameters ($p<0.05$).

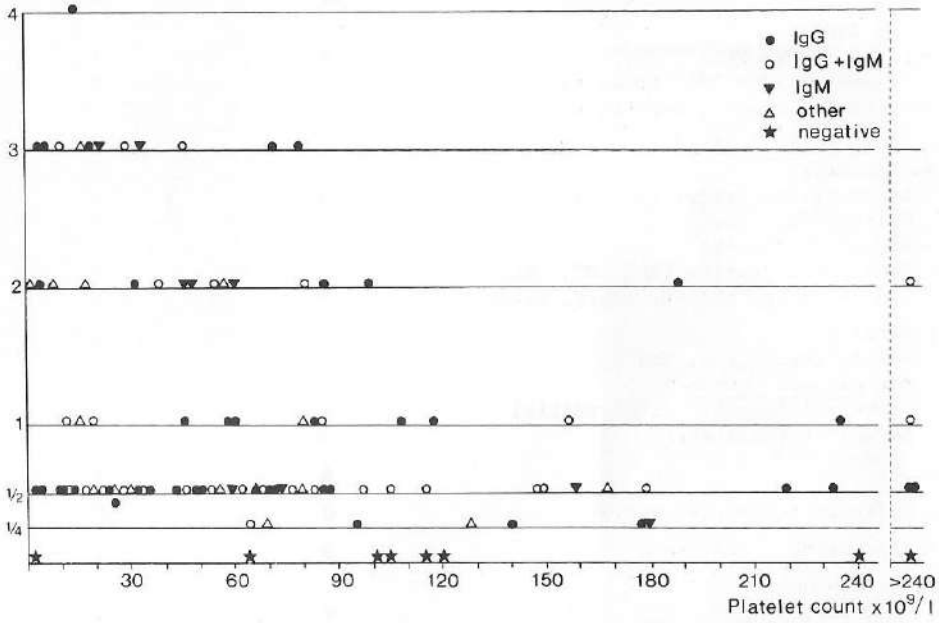

Fig. 1 - Relationship between the immunofluorescence strength, expressed as a score, and platelet count, as measured in the Coulter S plus II counter. Patients with platelet-bound autoantibodies of different Ig classes are depicted separately.

In the present series of ITP patients, a similar immunoglobulin-class distribution of the platelet-bound immunoglobulins and the eluted autoantibodies was found, as we have previously reported (Borne *et al*, 1980; Helmerhorst *et al*, 1982): i.e., IgG in 60 of the 67 (89.6%) patients tested (IgG alone in 44.8%), IgM in 33 of the 67 (49.3%) patients tested (IgM alone in 9.0%), IgA in 7 of the 67 (10.5%) patients tested (IgA alone in 0%). The Ig class of the autoantibodies was not related to the strength of

the direct test, or to the platelet count (depicted in Fig. 1). As previously reported, we could not detect complement fixation to the platelets for any patient.

TABLE II

Platelet immunofluorescence in thrombocytopenia
Results in 194 patients

	Number positive	Number negative
ITP	69	6
Other AID		
- Blood (AIHA, PCP)	5	1
- Organ-specific (SS, hepatitis, myositis, hyper-, hypothyroidism)	11	0
- Generalized (SLE, RA, FS)	9	0
Malignancies		
- Lymphoproliferative (CLL, NHL, ILA)	13	4
- Multiple myeloma	1	3
- Hodgkin's disease	0	3
- Myeloproliferative (AML, MD, CML, MH)	7	4
- Cancer (lung, breast, ovary, prostate)	9	1
Infection		
- viral (adenovirus, IM)	2	0
- mycoplasmal (pneumonia)	1	0
- bacterial (sepsis, endocarditis)	2	0
- parasitic (Kala Azar)	2	0
Drugs*	6	4
Hereditary thrombocytopenia	0	4
Pseudothrombocytopenia	3	0
Miscellaneous		
- Pregnancy	9	5
- TTP	1	0
- Liver cirrhosis (alcohol)	1	3
- Splenomegaly	1	0
- Vasculitis	1	2
- Diabetes	0	1
Total	153=78.9%	41=21.1%

Abbreviations: ITP, idiopathic thrombocytopenia; AID, autoimmune disease; AIHA, autoimmune hemolytic anemia; PCP, pancytopenia; SS, Sjögren syndrome; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; FS, Felty syndrome; CLL, chronic lymphatic leukaemia; NHL, non-Hodgkin's lymphoma; ILA, immunoblastic lymphadenopathy; AML, acute myeloid leukemia; MD, myelodysplasia; CML, chronic myeloid leukemia; MH, malignant histiocytosis; IM, infectious mononucleosis; TTP, thrombotic thrombocytopenic purpura.

* captopril, ibuprofen, gold, salazosulfapyridine, levodopa, benserazide, glibenclamide, mitomycin, trimethoprim, sulfametrol.

Diagnostic specificity of platelet immunofluorescence

The diagnoses of all 194 patients investigated, who were thrombocytopenic at the time of the tests, and the IFT results are shown in Table II. The diagnostic specificity of a positive test, calculated from these data for the diagnosis ITP, was low (29.4%), probably because positive test results were often found for patients with thrombocytopenia associated with other diseases. An overall prevalence of positive tests of 78.9% was found.

On the other hand, the negative predictive value for ITP of platelet immunofluorescence in the whole group of patients was high (85.4%), and even higher (96.6%) in patients with a low platelet count ($<100 \times 10^9/l$). This is a reflection of the rare occurrence of negative test results in ITP.

The occurence of EDTA- and PFA-dependent antibodies

When enough material was available, platelet eluates and sera from the patients of the present series, if positive in the standard tests, were also tested with unfixed donor platelets in EDTA buffer and with fixed donor platelets in PBS without EDTA. The results are shown in Table III.

TABLE III

The occurrence of EDTA- and PFA-dependent antibodies in eluates and sera from patients with positive test results in the standard method

	Eluate				Serum			
Diagnosis	Number tested	EDTA-dependent	PFA-dependent	Total	Number tested	EDTA-dependent	PFA-dependent	Total
ITP	41	0	0	0= 0 %	39	6	2	8=20.5%
Other TP's	33	6	0	6=18.2%	42	11	7	18=42.8%
ITP in remission	11	1	0	1= 9.1%	11	3	0	3=27.3%
No TP	6	3	0	3=50 %	6	3	3	6=100 %

The *eluates* of all 41 ITP patients that could be tested, contained antibodies reactive with unfixed donor platelets and fixed platelets without EDTA (EDTA- or PFA-independent antibodies). But in 8 out of 39 *sera* tested (20.5%), EDTA- or PFA-dependent antibodies were found. For the patients

with other thrombocytopenias, 6 of the 33 (18.2%) *eluates* contained EDTA-dependent antibodies and 18 of the 42 (42.8%) *sera* EDTA- or PFA-dependent antibodies. PFA antibodies were not detected in eluates. In this group of patients, there were three with pseudothrombocytopenia, containing EDTA antibodies in both eluate and serum.

EDTA-dependent antibodies were also detected in the platelet *eluates* from 1 of 11 (9.1%) patients with an ITP in remission and from 3 of 6 (50.0%) patients who had no thrombocytopenia, but who had a positive direct PIFT. The *sera* from these patients contained EDTA- and/or PFA-dependent antibodies in 3 out of 11 (27.3%) and 6 out of 6 (100%), respectively.

DISCUSSION

From our study, it appeared that the platelet immunofluorescence is a sensitive method to diagnose ITP, especially in patients with a platelet count of less than $100 \times 10^9/l$. Moreover, it seems likely that mostly, if not always, platelet autoantibodies and not platelet-bound complexes are responsible for ITP, because for most patients (93%) the platelet-bound immunoglobulins could be eluted by ether and detected in the eluate as platelet-reactive antibodies with normal donor platelets (Helmerhorst *et al*, 1984). A much higher percentage of positive results was obtained than in a previous study (von dem Borne *et al*, 1980), probably because a much better defined group of patients was investigated this time.

The degree of platelet autosensitization in ITP, as scored with the platelet immunofluorescence test, was often low: scores of ½-1 were found in about 60% of the patients (Fig. 1). This corresponds with amounts of platelet-bound immunoglobulins of less than 1 fg/platelet (or 3750 molecules/platelet) (Chapter II, Fig.5). These amounts are much lower than those detected by many investigators in quantitive antiglobulin consumption assays (von dem Borne *et al*, 1984) except those found in recent studies of LoBuglio *et al* (1983, 1984). As already discussed, this discrepancy may be explained by the interference of platelet fragments in many of these assays.

Similarly to results of the various antiglobulin consumption assays, for instance those of Kelton *et al* (1982), a positive immunofluorescence test result had a low specificity for the diagnosis of ITP. Our present investigations show that this low specificity may have two causes. First,

autoimmune thrombocytopenia, with platelet autosensitization detected by the PIFT, occurs together with many other diseases (Helmerhorst *et al*, 1982) (Table III). Second, in the blood of the patients, antibodies may exist that are directed against hidden or cryptic antigens of platelets (von dem Borne *et al*, 1986). These antibodies react probably with antigenic determinants of the glycoprotein IIb-IIIa complex, which are exposed under the influence of Ca^{++}-chelating agents, such as EDTA and EGTA, or after paraformaldehyde fixation. These antibodies have been detected not only in the blood of patients with pseudothrombocytopenia (Pegels *et al*, 1982), with septicemia (van der Lelie *et al*, 1984) and in uremic patients on hemodialysis (Magee, 1985), but also in patients with a variety of other diseases, both with and without thrombocytopenia, such as autoimmune thrombocytopenia (primary and secondary), myelodysplasia, toxemia of pregnancy and thrombotic thrombocytopenic purpura (von dem Borne *et al*, 1986). This prevalence was also reflected in the present series of patients.

It seems unlikely that antibodies against cryptantigens of platelets are responsible for platelet destruction *in vivo*, but they could be the results of it (van der Lelie *et al*, 1984; von dem Borne *et al*, 1986). Binding of these antibodies probably occurs only *in vitro*, due to anticoagulation of the patient's blood with EDTA or fixation of the platelets with paraformaldehyde. For patients with a manifest ITP, platelet eluates always contained antibodies that reacted with unaltered platelets (unfixed, in the absence of EDTA). Probably, these antibodies are the pathogenetically important ones. But 8 of the 39 ITP sera contained (only) cryptantigen antibodies*. They were sometimes found in platelet eluates from patients with ITP in remission, but much more frequently in platelet eluates from patients with thrombocytopenias other than ITP and from patients with diseases without thrombocytopenia but with a positive direct immunofluorescence test. The sera of such patients contained (only) cryptantigen antibodies in a high frequency (40-100%). Hence, this type of antibody contributes to the low diagnostic specificity of the platelet immunofluorescence test.

---

*The co-occurrence of antibodies reactive with unaltered antigens and antibodies against cryptantigens in eluates and sera was not studied systematically. But in some cases, it was found to exist by way of absorption and elution studies.

Cryptantigen antibodies may also be detected in other platelet antibody tests, such as the quantitative antiglobulin consumption assays. These antibodies may be responsible for the positive PAIgG results found for patients in whom the autoimmune nature of the thrombocytopenia is doubtful, such as in septicemia, toxemia of pregnancy, liver cirrhosis and hypersplenism (Kelton *et al*, 1982, 1985; van der Lelie *et al*, 1981). Their presence may even explain the finding of an increased amount of platelet-associated IgG in patients with a normal platelet count and platelet life-span (Kelton *et al*, 1984).

In conclusion, platelet immunofluorescence has a high sensitivity in the detection of platelet autosensitization in ITP, but it has a low specificity for this diagnosis. The low specificity is caused not only by the co-occurrence of autoimmune thrombocytopenia and other diseases, but also by the occurrence of antibodies against platelet cryptantigens in the blood of many patients. Such antibodies may be detected not only in the immunofluorescence test, but in other platelet antibody tests as well.

ACKNOWLEDGEMENT

We thank all the clinicians who so kindly provided us with the clinical data from their patients.

REFERENCES

BORNE, A.E.G. Kr. von dem, VERHEUGT, F.W.A., OOSTERHOF, F., RIESZ, E. von, BRUTEL de la RIVIERE, A. & ENGELFRIET, C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. *British Journal of Haematology*, 39, 195-207.

BORNE, A.E.G. Kr. von dem, HELMERHORST, F.M., LEEUWEN, E.F. van, PEGELS, J. G., RIESZ, E. von & ENGELFRIET, C.P. (1980) Auto-immune thrombocytopenia: detection of platelet auto-antibodies with the suspension immunofluorescence test. *British Journal of Haematology*, 45, 319-327.

BORNE, A.E.G. Kr. von dem. (1984) Autoimmune thrombocytopenia. In: *Research Monographs in Immunology*, volume 5, 'Immunohaematology'. Engelfriet, C.P., van Loghem, J.J. & von dem Borne, A.E.G. Kr. (eds.). Elsevier Science Publishers, pp 222-257.

BORNE, A.E.G. Kr. von dem, LELIE, J. van der, VOS, J.J.E., PLAS-van DALEN, C.M. van der, RISSEEUW-BOGAERT, N.J., TICHELER, M.D.A. & PEGELS, J.G. (1986) Antibodies against cryptantigens of platelets. Characterization and significance for the serologist. In: *Platelet Serology, Research Progress and Clinical Implications*. Decary, F. & Rock, G.A. (eds). Karger, Basel, pp 33-46.

HELMERHORST, F.M., BOSSERS, B., BRUIN, H.G. de, ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1980) Detection of platelet antibodies: a comparison of three techniques. *Vox Sanguinis*, 39, 82-83.

HELMERHORST, F.M., LEEUWEN, E.F. van, PEGELS, J.G., PLAS-van DALEN, C.M. van der, ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1982) Primary and secondary autoimmune thrombocytopenia. A serological and clinical analysis. *Scandinavian Journal of Haematology*, 28, 319-328.

HELMERHORST, F.M., SMEENK, R.T.J., HACK, C.E., ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1983) Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies. *British Journal of Haematology*, 55, 533-545.

KELTON, J.G., POWERS, P.J. & CARTER, C.J. (1982) A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. *Blood* 60, 1050-1053.

KELTON, J.G., CARTER, C.J., RODGER, C., BEBONEK, G., GAULDIE, J., SHERIDON, D., KASSAM, Y.B., KAEN, W.F., BUCHANAN, W., ROONAY, P.J., BIANCHI, F. & DENBURG, J. (1984) The relationship among platelet-associated IgG, platelet life span and reticuloendothelial cell function. *Blood*, 63, 1434-1438.

KELTON, J.G. (1985) Platelet autoantibodies and septicaemia. *British Journal of Haematology*, 61, 589-590.

LELIE, J. van der, PLAS-van DALEN, C.M. van der & BORNE, A.E.G. Kr. von dem (1984) Platelet autoantibodies in septicaemia. *British Journal of Haematology*, 58, 755-760.

LELIE, J. van der, PLAS-van DALEN, C.M. van der & BORNE, A.E.G. Kr. von dem (1985) Platelet autoantibodies and septicaemia. Letter to the Editor. *British Journal of Haematology*, 61, 591-593.

LoBUGLIO, A.F., COURT, W.S., VINOCUR, L., MAGLOTT, G. & SHAN, G.M. (1983) Immunothrombocytopenic purpura. Use of a ^{125}I-labelled anti-human IgG monoclonal antibody to quantify platelet-bound IgG. *New England Journal of Medicine*, 309, 459-463.

MAGEE, J.M. (1985) A study on the effects of treating platelets with paraformaldehyde for use in the platelet suspension immunofluorescence test. *British Journal of Haematology*, 61, 513-516.

MUELLER-ECKHARDT, C., KAYSER, W., MERSH-BAUMERT, K., MUELLER-ECKHARDT, G., BREIDENBACH, M., LUGEL, H.G. & GRAUBIER, M. (1980) The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. *British Journal of Haematology*, 46, 123-131.

MUELLER-ECKHARDT, C., MUELLER-ECKHARDT, G., KAYSER, W., VOSS, R.M., WEGNER, J. & KUENZLER, E. (1982) Platelet-associated IgG, platelet survival and platelet sequestration in thrombocytopenic states. *British Journal of Haematology*, 52, 49-58.

PEGELS, J.G., BRUYNES, E.C.E., ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem (1982) Pseudo-thrombocytopenia: an immunologic study on platelet antibodies dependent on ethylene diamine tetra-acetate. *Blood*, 59, 157-161.

SHAW, G.M., AXELSON, J., MAGLOTT, J.G. & LoBUGLIO, A.F. (1984) Quantification of platelet-bound IgG by ^{125}I-staphylococcal protein A in immune thrombocytopenic purpura and other thrombocytopenic disorders. *Blood*, 63, 154-161.

SHULMAN, N.R., LEISSINGER, C.A., HOTCHKISS, A.J. & KAUTZ, C.A. (1982) The non-specific nature of platelet-associated IgG. *Transactions Association American Physicians*, 14, 213-220.

VOS, J.J.E., HUISMAN, J.G., WINKEL, I.N., RISSEEUW-BOGAERT, N.J., ENGELFRIET, C.P. & BORNE, A.E.G. Kr. von dem. (1986) Quantification of platelet-bound alloantibodies by radioimmunoassay. A study on some variables. Submitted for publication.

CHAPTER IV

PLATELET-ASSOCIATED IgG IN THROMBOCYTOPENIA.
A comparison of two techniques

J.J.E. Vos, J.G. Huisman, J. van der Lelie and A.E.G. Kr. von dem Borne

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and the Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands.

SUMMARY

The results obtained in the analysis of 130 thrombocytopenic patients with a radioimmunoassay (RIA) for platelet-associated IgG (PA-IgG) and the platelet suspension immunofluorescence test (PIFT) were compared. The RIA was positive in 33 of 41 (82.9%) patients with idiopathic thrombocytopenia (ITP) and in 51 of 79 (64.4%) patients with secondary thrombocytopenia (STP). The PIFT was positive in 37 of the 41 (90.2%) ITP patients and in 57 of the 79 (72.2%) STP patients. Sensitivity and specificity for the diagnosis of ITP of both tests were comparable: 82.9% and 40.9% for the RIA, and 90.2% and 36.7% for the PIFT.

A significant positive correlation was observed between the mean amount of PA-IgG measured and the height of PIFT scores with anti-IgG. A more frequent incidence of aberrant size distribution patterns was found in patients with increased PA-IgG, indicating a possible influence of platelet fragments on the measurement of PA-IgG.

In patients with increased PA-IgG and a negative PIFT with anti-IgG, IgM antibodies were detected more frequently in the PIFT. This suggests an influence on PA-IgG determinations of either IgM antibody-induced platelet agglutination *in vitro* or of platelet fragments present in the patient's blood because of IgM antibody-mediated platelet destruction *in vivo*.

Submitted for publication

INTRODUCTION

Many assays for the detection of platelet-associated IgG (PA-IgG) have been described in the literature to establish the presumed autoimmune nature of idiopathic thrombocytopenia (ITP). These tests vary considerably in the obtained values of PA-IgG measured in normal donors (0.1 - 26.0 fg/platelet), as well as in patients (0.1-1800 fg/platelet) (McMillan, 1981; von dem Borne, 1984). The values detected in normal donors are often higher than is to be expected from the number (200 - 4000) of Fc receptors on the platelet surface (Karas *et al*, 1982).

Moreover, often unexpectedly increased PA-IgG values are detected, e.g. in patients with many different non-immune-mediated diseases, even without thrombocytopenia (Mueller-Eckhardt *et al*, 1980). The occurrence of platelet fragments and/or aggregates have been proposed as possible causes for this phenomenon (Shulman *et al*, 1982). Both fragments and aggregates escape from accurate platelet counting, whereas their contribution to the total IgG content remains. Therefore, falsely elevated PA-IgG values may be measured.

Recently, we have developed a radioimmunoassay for PA-IgG, which allows accurate measurement of platelet-bound alloantibodies (Vos *et al*, submitted for publication). Platelet fixation with paraformaldehyde (PFA) appeared to accomplish a loss of non-specific PA-IgG. Normal donors had low amounts of PA-IgG compared to amounts measured by most other tests reported (0.3 ± 0.3 fg/platelet, m ± 2 sd).

In this report, we compare the results of the radioimmunoassay with those of the direct platelet suspension immunofluorescence test (PIFT) (von dem Borne *et al*, 1980) in a group of 130 thrombocytopenic patients, whose blood was sent to our laboratory for platelet-antibody tests.

MATERIALS AND METHODS

Isolation of platelets

Platelet-rich plasma was separated from blood collected in 10-ml tubes containing 4 mM EDTA (venoject). Erythrocytes and leukocytes were pelleted by centrifugation during 10 min at 350 g. Platelets were pelleted from the PRP by centrifugation for 7 min at 2000 g and were washed 3 times in a solution of Na_2EDTA (9 mM) in phosphate-buffered saline, pH 7.4 with 0.2% (w/v) bovine serum albumin (EDTA-PBS-EDTA). The pelleted washed platelets were resuspended in 1 ml of a solution of 1% (w/v) PFA in PBS for 5 min at room temperature, washed again twice, resuspended in EDTA-PBS-BSA, counted in a Coulter ZF automatic blood counter and adjusted to a concentration of $5x10^7$/ml.

Platelet suspension immunofluorescence test (PIFT)

This test has been described in detail (von dem Borne *et al*, 1978,1980). In brief, platelets were isolated, fixed with PFA and then tested directly for platelet-bound antibodies with FITC-labelled antiglobulin sera (sheep-anti-human Ig prepared in our laboratory, rabbit anti-IgG, -IgA and -IgM purchased from Dakopatts, Denmark, appropriately diluted in PBS-0.2% (w/v) BSA). Subsequently, the platelets were washed twice, resuspended in 50% (v/v) glycerol in PBS and examined under the fluorescence microscope. Results were expressed as -, ±, (+), 1+, 2+, 3+, 4+ (scored as 0, 0.25, 0.5, 1, 2, 3, 4, respectively). Also, to confirm the autoantibody nature of the platelet-bound immunoglobulins, they were eluted with diethyl ether (von dem Borne *et al*, 1980), and subsequently tested for their reactivity with donor platelets.

Radioimmunoassay for PA-IgG

One hundred µl of a suspension of PFA-treated platelets were incubated with 100 µl of ^{125}I-radiolabelled rabbit-anti-human IgG $F(ab')_2$ fragments for 16 h at 4°C in a polystyrene tray (Abbott Diagnostic Products, USA). A polystyrene bead (Spherotech, diameter 6.35 mm) coated with human IgG was then added to the reaction mixture and the incubation was allowed to continue for 6 h at room temperature. Finally, the beads were washed 4 times with distilled water, transferred to counting tubes and bound radioactivity was measured in a gamma scintillation counter.

Five dilutions of a platelet suspension (range from 50 - 25 - 12.5 - 6.25 - $3.13x10^6$/ml) were tested in triplicate. Simultaneously, a standard IgG reference curve (Cohn fraction II) was prepared in triplicate with a range between 7.8 and 62.5 ng human IgG/ml.

The percentage inhibition of radioactivity (RA) bound to the IgG-coated bead by the test sample was calculated from the formula:

$$\% \text{ inhibition} = \left(1 - \frac{\% \text{ RA unbound by test sample}}{\% \text{ RA unbound by blank sample}}\right) \times 100\%$$

The amount of IgG corresponding to this percentage inhibition was directly read from the calibration curve and was subsequently correlated to the number of platelets known to be present in the test sample. In general, 3 or 4 dilutions of a platelet suspension appeared to have IgG values within the range of the calibration curve. According to these measurements, the average PA-IgG value was calculated and expressed as fg/platelet.

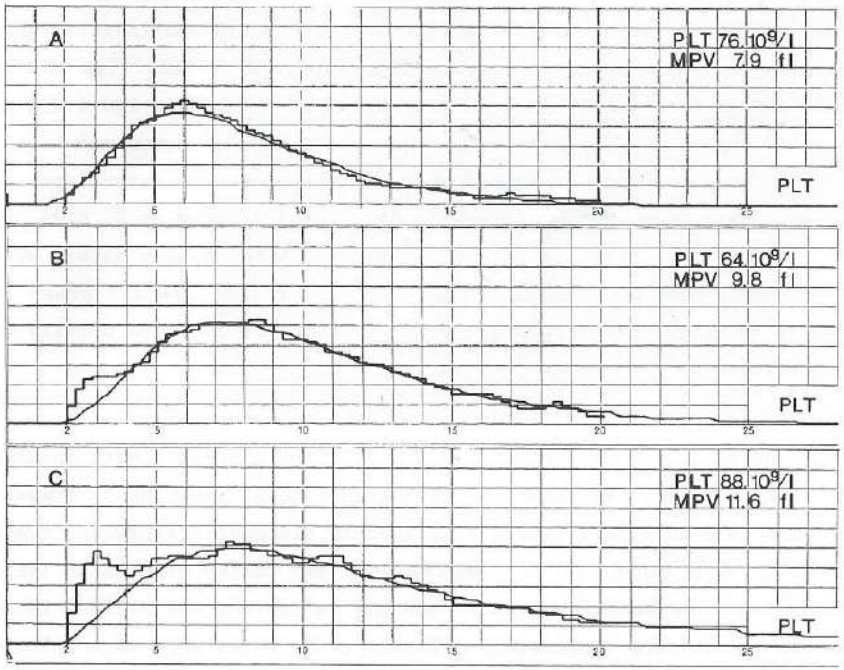

Fig. 1A, B, C

Fig. 1 - Various patterns of platelet size distribution plots obtained by measurement on Coulter counter S plus II. Pattern A is normal and pattern B is nearly normal. Patterns C-F represent abnormal size distribution patterns.

Coulter counter determinations

EDTA-anticoagulated blood samples of the patients were analysed by a Coulter counter, model S plus II, yielding platelet counts, mean platelet volume and size distribution curves. Size distribution curves were scored as described by van der Lelie *et al* (submitted for publication). Patients could be divided into two groups: those having normal or nearly normal curves (patterns A and B), probably containing no or very low numbers of fragmented cells, and those having abnormal size distribution curves (patterns C-F), probably containing fragments of platelets (Fig. 1).

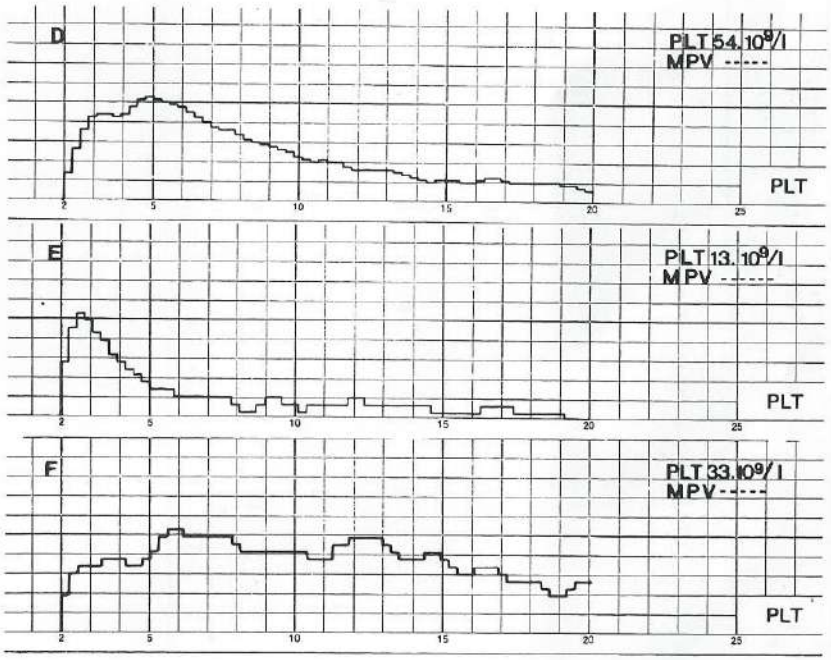

Fig. 1D, E, F

Patients

We examined blood samples of 130 thrombocytopenic patients (44 men, 86 women; mean age 47 years; platelet count $<150\times10^9/l$). Samples were sent to us by physicians from all over the country in the period between September 1, 1984 and January 1, 1985. Only samples containing sufficient amounts of platelets to perform both tests were investigated. Clinical information was obtained by a questionnaire and inadequate information was completed by inquiries by telephone.

TABLE I

Results of the PIFT and the PA-IgG assay in various groups of patients

Diagnosis	Total number of patients	PIFT with anti- -Ig	-IgG	-IgA	-IgM	PA-IgG elevated	Discrepancies: Only PA-IgG elevated positive	Only PIFT-IgG positive
I. ITP	41	37 (90.2%)	20 (48.8%)	4 (9.8%)	28 (68.2%)	33 (80.5%)	14 (34.1%)	1 (2.4%)
II. Thrombocytopenia accompanied by:								
a. autoimmune diseases (blood, generalized, localized)	20	16	10	5	12	15	5	0
b. malignancies (lymphoid, myeloid, solid)	18	14	10	1	10	13	3	0
c. infectious disease (viral, bacterial, parasital)	9	8	4	0	5	7	3	0
d. medicamenteous	10	8	6	0	5	6	1	1
e. various other diseases	22	11	6	4	8	10	4	0
Total	79	57 (72.2%)	36 (45.6%)	10 (12.7%)	40 (50.6%)	51 (69.6%)	16 (20.2%)	1 (1.3%)
III Bone marrow disease (infiltration, cytostatics/ irradiation, hypoplasia)	10	7 (70.0%)	6 (60.0%)	1 (10.0%)	4 (40.0%)	6 (60.0%)	3 (30.0%)	3 (30.0%)

The patients could be divided into three groups. Group 1 contained 41 patients (17 men, 24 women; mean age 33 years) fulfilling the diagnostic criteria for idiopathic thrombocytopenic purpura (ITP). Group 2 consisted of 79 patients (22 men, 57 women; mean age 53 years) who had thrombocytopenia with the above clinical features, but accompanied by other diseases, which were often of autoimmune origin (secondary thrombocytopenia, STP). Amongst them were 9 patients with a proved preceding infectious disease, 4 patients with autoimmune blood disorders, 11 patients with generalized autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosis), 4 with an autoimmune disorder confined to one organ. Fourteen patients were suffering from lymphatic or myeloid malignancies and 3 from a solid tumor, none of whom were treated with cytostatic and/or irradiation therapy. Eight patients had drug-induced thrombocytopenia without bone-marrow suppression, whereas 9 patients had thrombocytopenia accompanied by other diseases (liver cirrhosis, toxaemia of pregnancy). None of the patients of group 2 was treated with cytostatic therapy. Group 3 contained 10 patients (5 men, 5 women; mean age 60 years) who had thrombocytopenia due to bone-marrow failure (cytostatic and/or irradiation therapy, hypoplasia). For more details, see Table I.

RESULTS

Platelet-associated IgG (PA-IgG) as measured by the radioimmunoassay was elevated in 90 of the 130 thrombocytopenic patients (69.2%). Platelet-bound immunoglobulins (PBIg) as observed by direct immunofluorescence were present on platelets of 101 patients (77.7%), the eluate being positive in 91 patients (70.0%). The Ig class of the PBIg detected in the direct PIFT was found to be IgG in 62 patients (47.7%), IgM in 71 patients (54.6%) and IgA in 15 patients (11.5%) (Table I).

In 57 patients, PA-IgG was elevated and the PIFT with anti-IgG (PIFT-IgG) was positive, in 33 patients only PA-IgG was elevated, whereas in 5 patients only the PIFT-IgG was positive. Results of the two tests correlated well ($p<0.001$; χ^2 test). Moreover, although in this study no linear correlation between results of the two tests was observed ($p<0.25$; Kruskal-Wallis test), the mean PA-IgG increased as the PIFT-IgG was scored higher (PIFT score 0: m ± s.d. = 1.2 ± 1.3 fg/platelet; PIFT score 0.25-0.5: 1.7 ± 1.7 fg/platelet, $p<0.05$ Student's t-test; PIFT score 1-4: 5.1 ±

5.0 fg/platelet, p<0.005 Student's t-test) (Fig. 2).

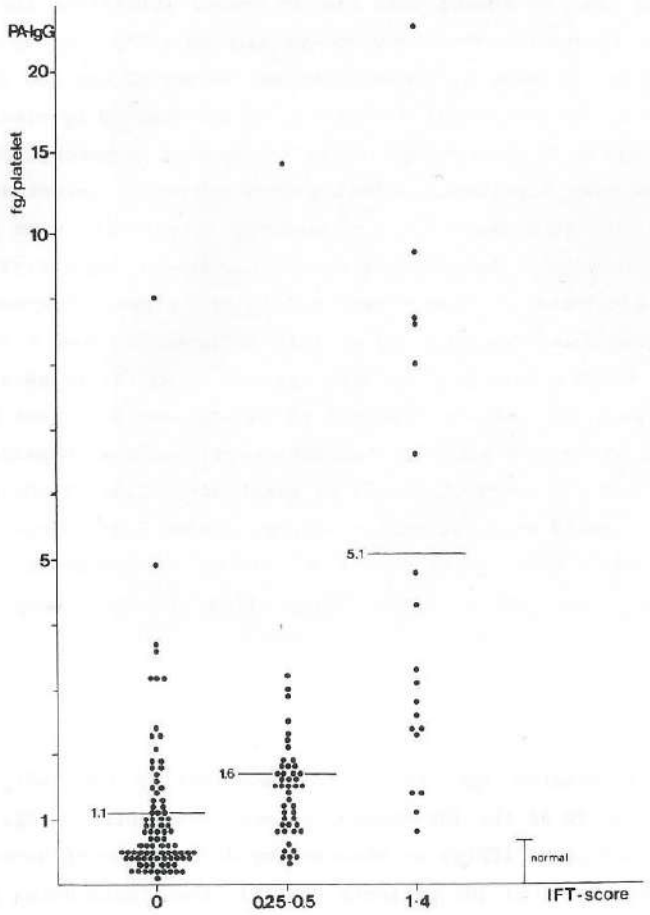

Fig. 2 - Relationship between results of PA-IgG and PIFT-IgG in thrombocytopenic patients. Mean PA-IgG increased as PIFT-IgG was scored higher (p< 0.005, Student's t-test).

IgM antibodies

In patients with an elevated PA-IgG value and a negative PIFT-IgG, a positive PIFT with anti-IgM occurred significantly more often than in all other patients (62.5 and 38.8%, respectively, p<0.05, χ^2 test) (Table II).

Platelet count

Although no linear correlation was observed, the platelet count was inversely related to the amount of PA-IgG as measured in the RIA (p<0.01,

TABLE II

Relationship between results of PIFT-IgG and PA-IgG in thrombocytopenic patients

Group	Number of patients	Pattern A	B	C-F	Percentage occurrence of C-F patterns	Percentage of patients with IgM antibodies
+/+ both positive	57	8	14	29	56.9	35.1
+/- only PA-IgG elevated	33	6	3	17	65.4	60.6
-/+ only PIFT-IgG positive	5	2	2	1	20.0	20
-/- both tests negative	35	16	9	7	21.9	48.6

In groups I and II and overall, a significant correlation was observed between positivity in PA-IgG and PIFT-IgG (p<0.0001, χ^2 test). In patients with an elevated PA-IgG, an abnormal size distribution pattern was observed significantly more often (p<0.001, χ^2 test). In patients with an elevated PA-IgG and a negative PIFT-IgG, IgM antibodies were found significantly more often, whereas only four patients in this group had no IgM antibodies and a normal or nearly normal size distribution pattern.

Student's t-test), as well as to the score in the PIFT with anti-IgG (p< 0.05, χ^2 test).

Size distribution plots

Size distribution plots were obtained from blood samples of 114 patients. Fifty five patients had abnormal patterns (C-F) (cf. Fig. 2), indicating circulating fragments. In patients with elevated PA-IgG and negative PIFT-IgG, abnormal size distribution plots were observed significantly more often than in patients with normal PA-IgG and negative PIFT-IgG (65.4 and 21.9%, respectively; p<0.001 χ^2 test) (Table II). Moreover, in the group of patients with elevated PA-IgG and a negative PIFT-IgG, abnormal size distribution curves were more frequently seen than in the group of patients with both an elevated PA-IgG and a positive PIFT-IgG. However, this difference was not significant (p>0.05, χ^2 test).

Relationship to diagnosis

In Table I and Fig. 3, results of the PA-IgG test and the PIFT in various diseases are depicted. In patients with ITP (group 1), the PIFT was

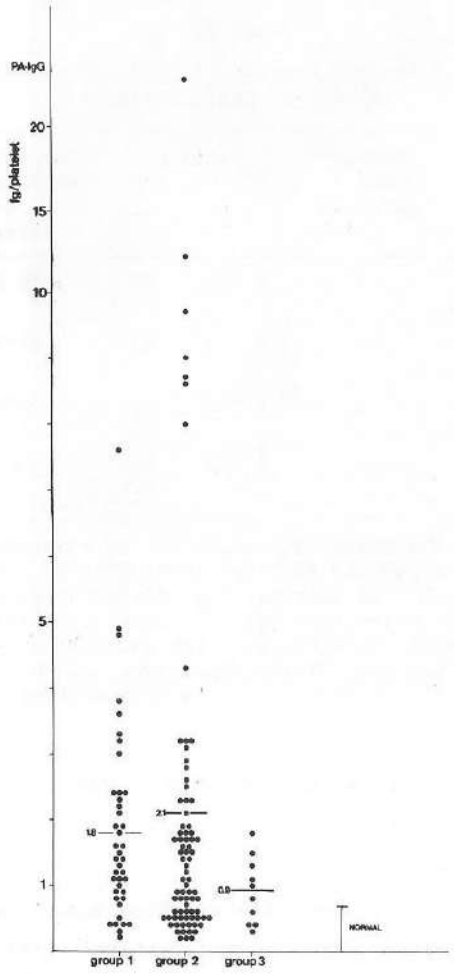

Fig. 3 - PA-IgG results in various groups of patients. Group 1, ITP patients; group 2, patients with thrombocytopenia combined with other diseases (STP patients); group 3, patients with bone-marrow suppression by irradiation, treatment with cytostatics or with bone-marrow infiltration or hypoplasia (see also Materials and Methods and Table I).

positive in 37/41 patients with ITP (90.2%) of whom 20 were positive with anti-IgG (48.8%). The specificity of the PIFT for the diagnosis of ITP was 36.7%. PA-IgG was elevated in 33/41 ITP patients (82.9%), the mean PA-IgG being 1.8±1.4 fg/platelet (m ± s.d.). The specificity of the PA-IgG test for the diagnosis of ITP was 40.9%.

DISCUSSION

Advantages of the RIA as compared to the PIFT are its quantitative character and the fact that smaller quantities of patients' blood are needed. The main advantage of the PIFT is that, upon microscopic examination, only intact platelets are examined by which the influence of platelet fragments and/or aggregates is excluded. The two techniques are equally simple to perform.

In their study, published in 1980, Kelton *et al* observed an overall higher sensitivity and specificity of their antiglobulin consumption assay (94 and 95%, respectively) than of the immunofluorescence test (44 and 82%, respectively). Later, the specificity of the same antiglobulin consumption assay was reported to be about 50% (Kelton *et al*, 1982). In our present study, we found, in contrast to Kelton *et al*, that the sensitivity and specificity of both tests for the diagnosis of ITP were about the same. Moreover, we found that, although there was no linear correlation, mean PA-IgG increased as the score of the PIFT was higher. The differences between our results and those of Kelton's 1980 study may be explained by the fact that Kelton *et al* selected their patients differently and that the PIFT was only carried out with an anti-IgG conjugate.

In contrast to previous studies of our laboratory (von dem Borne *et al*, 1980; Helmerhorst *et al*, 1982; von dem Borne *et al*, submitted for publication), more often IgM antibodies and less often IgG antibodies were detected in the present one. Selection of patients for sufficient numbers of platelets in the blood to perform both tests or perhaps a seasonal influence may have caused this discrepancy.

Despite the above-mentioned relationship between results of the PA-IgG test and the PIFT with anti-IgG (PIFT-IgG), discrepancies between results of the two tests were quite often found as well. In 33 of the 130 thrombocytopenic patients PA-IgG was elevated, whereas the PIFT-IgG was negative; while in 5 patients only the PIFT-IgG was positive and PA-IgG was not elevated.

Various explanations may be offered for these discrepancies. Borderline results were often seen in this group (dubious or weakly positive results in the PIFT or the PA-IgG test, i.e. 0.8 - 1.0 fg/platelet). All negative RIAs (n=5) with a positive PIFT-IgG could be ascribed to this, as well as 10 of the 33 positive RIAs with a negative PIFT-IgG.

Also, an elevated amount of PA-IgG with a negative PIFT might be explained by the presence of platelet agglutinates and/or fragments (Shulman *et al*, 1982) in the test samples. Both agglutinates and fragments escape from accurate platelet counting. However, their contribution to the total IgG content remains and, therefore, a falsely elevated PA-IgG value may be measured (Vos *et al*, submitted for publication).

In our present study, we found that platelet autoantibodies of the IgM class, as detected in the PIFT with anti-IgM, occurred significantly more often in the blood of patients with a negative PIFT with anti-IgG and an elevated PA-IgG, compared to patients positive in both assays. Because platelet agglutinins are of the IgM subclass (van der Giessen *et al*, 1964; van der Weerdt, 1965), it is possible that the IgM-platelet autoantibodies caused platelet agglutination *in vitro* (which was noticed quite often microscopically, but not studied systematically) and, in this way, elevated PA-IgG values. However, it is also possible that the IgM autoantibodies caused an elevated PA-IgG via the induction of platelet destruction and fragmentation *in vivo*, resulting in significant amounts of platelet fragments in the patient's blood. Indeed, irregular platelet-size distribution plots, suggestive for the occurrence of circulating fragments (Kahn *et al*, 1975) were significantly more often observed in patients with an increased PA-IgG value, compared to those with a normal value, especially when the PIFT with anti-IgG was negative.

A factor not systematically tested in the present study was the occurrence of EDTA-dependent platelet antibodies that have been found in pseudothrombocytopenia (Pegels *et al*, 1982), in septicaemia (Van der Lelie *et al*, 1984), but also in many other thrombocytopenic and non-thrombocytopenic disorders and even in ITP (Von dem Borne *et al*, 1986). Such antibodies are often of the IgM subclass and may cause platelet agglutination. In fact, these antibodies could often be the IgM antibodies detected in the patient's blood discussed above and, therefore, be an important source for aspecifically elevated PA-IgG values, because EDTA blood is used by most investigators (including ourselves) for PA-IgG measurement.

In conclusion, PIFT and RIA for PA-IgG have about the same (high) sensitivity and the same (low) specificity for the diagnosis of ITP. There was good correlation between the results of the two tests performed on platelets of thrombocytopenic patients, although this relationship was not direct. Most discrepancies between PA-IgG and PIFT may be explained by either borderline results or by the occurrence of agglutinates induced by IgM antibodies and/or fragments of platelets in the test samples. Further investigations will be necessary to determine relative roles of each of these phenomena as well as the contribution of the occurrence of EDTA-dependent antibodies. A more systematic study into these variables is presently being undertaken in our laboratory.

ACKNOWLEDGEMENTS

All staff of the Department for Platelet and Granylocyte Serology is acknowledged for their technical assistence, especially Mrs N.J. Risseeuw-Bogaert, Ms B. Bossers, Ms I. Damsma and Ms M. Nap for performing the radioimmunoassay for PA-IgG. Also, the authors thank Mr. W. Schaasberg for statistical advice, Mr. H.L. Zaaijer for help in data processing and Ms S. Scholten, Mrs W. Winkel-Groeneveld and Mrs J. Gerritsen for secretarial help.

REFERENCES

BORNE, A.E.G. Kr. Von dem, VERHEUGT, F.W.A., OOSTERHOF, F., RIESZ, E. Von, BRUTEL de la RIVIERE, A. & ENGELFRIET, C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. *British Journal of Haematology*, 39, 195-207.

BORNE, A.E.G. Kr. Von dem, HELMERHORST, F.M., LEEUWEN, E.F. Van, PEGELS, J.G., RIESZ, E. Von & ENGELFRIET, C.P. (1980) Autoimmune thrombocytopenia: detection of platelet autoantibodies with the suspension immunofluorescence test. *British Journal of Haematology*, 45, 319-327.

BORNE, A.E.G. Kr. Von dem. (1984) Autoimmune thrombocytopenia. In *Immunohaematology*, Engelfriet, C.P., Loghem, J.J. & Borne, A.E.G. Kr. von dem, eds., Elsevier, Amsterdam, pp 222-256.

BORNE, A.E.G. Kr. Von dem, LELIE, J. Van der, VOS, J.J.E., PLAS-van DALEN, C.M. Van der, RISSEEUW-BOGAERT, N.J., TICHELER, M.D.A. & PEGELS, J.G. (1986) Antibodies against cryptantigens of platelets. Characterization and significance for the serologist. In *Platelet Serology, Research Progress and Clinical Implications*, Décary, F. and Rock, G.A., eds., Karger, Basel, pp 33-46.

GIESSEN, M. Van der, HART, M. Van der & WEERDT, C.M. Van der (1964) Fractionation of sera containing antibodies against red cells or platelets with special reference to anti-D sera. *Vox Sanguinis*, 9, 25-30.

HELMERHORST, F.M., LEEUWEN, E.F. Van, PEGELS, J.G., PLAS-van DALEN, C.M. Van der, ENGELFRIET, C.P. & BORNE, A.E.G. Kr. Von dem (1982) Primary and secondary autoimmune thrombocytopenia: a serological and clinical analysis. *Scandinavian Journal of Haematology*, 28, 319-328.

KARAS, S.P., ROSSE, W.F. & KURLANDER, R.J. (1982) Characterization of the IgG-Fc receptor on human platelets. *Blood*, 60, 1277-1282.

KELTON, J.G., GILES, A.R., NEAME, P.B., POWERS, P., HAGEMAN, N. & HIRSCH, J. (1980) Comparison of two direct assays for platelet-associated IgG (PA-IgG) in assessment of immune and non-immune thrombocytopenia. *Blood*, 55, 424-429.

KELTON, J.G., POWERS, P.J. & CARTER, C.L. (1982) A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. *Blood*, 60, 1050-1053.

KHAN, I., ZUCKER-FRANKLIN, D. & KARPATKIN, S. (1975) Microthrombocytosis and platelet fragmentation associated with idiopathic/autoimmune thrombocytopenia. *British Journal of Haematology*, 31, 449.

LELIE, J. Van der, PLAS-van DALEN, C.M. Van der & BORNE, A.E.G. Kr. Von dem (1984) Platelet autoantibodies in septicaemia. *British Journal of Haematology*, 58, 755-760.

McMILLAN, R. (1981) Chronic idiopathic thrombocytopenic purpura. *New England Journal of Medicine*, 304, 1135-1147.

MUELLER-ECKHARDT, C., KAYSER, W., MERSCH-BAUMUST, R., MUELLER-ECKHARDT, G., BREIDENBACH, M., KUGEL, H.G. & GRAUBOER, M. (1980) The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. *British Journal of Haematology*, 46, 123-131.

PEGELS, J.G., BORNE, A.E.G. Kr. Von dem, BRUYNES, E.C.E. & ENGELFRIET, C.P. (1982) Pseudothrombocytopenia: an immunological study on platelet antibodies dependent on ethylene diamine tetra-acetate. *Blood*, 59, 157-161.

SHULMAN, N.R., LEISINGER, C.A., HOTCHKIN, A.J. & KANTZ, C.A. (1982) The nonspecific nature of platelet-associated IgG. In *Transactions of the Association of American Physicians*, Vol. XCV, 213-220.

WEERDT, C.M. Van der. (1965) Platelet antigen and iso-immunization. Thesis, Amsterdam, Aemstelstad.

CHAPTER V

DETECTION OF CIRCULATING HUMAN PLATELET FRAGMENTS BY USING MONOCLONAL ANTIBODIES AGAINST PLATELET GLYCOPROTEIN IIb-IIIa COMPLEX

by J.J.E. Vos, J.G. Huisman, P.W. Modderman and A.E.G. Kr von dem Borne

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, incorporating the Laboratory of Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands

SUMMARY

A radioimmunoassay (RIA) for the detection of platelets and platelet fragments was developed. A sandwich of two monoclonal antibodies directed against the platelet-specific glycoprotein complex IIb-IIIa (GPIIb-IIIa) was used in this assay. A discontinuous 7.5-20% (v/v) albumin gradient was applied to separate platelets of various sizes as well as fragments derived thereof. In platelet suspensions fractionated in this way, we observed that particles smaller than normal platelets still carried the GP IIb-IIIa antigens. This procedure enabled us to detect platelet-derived particles in platelet-rich plasma from thrombocytopenic patients.

Submitted for publication

INTRODUCTION

The occurrence of *in-vivo* platelet fragmentation in patients with immune thrombocytopenia has been demonstrated by ultracentrifugation and electron microscopy, as well as by studies on the size distribution of platelets as determined by a Coulter Channelyzer (Khan *et al*, 1975; Zucker-Francklin & Karpatkin, 1977; George *et al*, 1982; Shulman *et al*, 1982).

The presence of platelet fragments in the blood of patients with thrombocytopenia is of interest, both for clinicians and serologists. It could be used as a parameter for increased (*in-vivo*) platelet destruction. Moreover, it could offer an explanation for false-positive results of assays for platelet-associated IgG (Shulman *et al*, 1982; Vos *et al*, submitted). Therefore, we developed an assay for the detection of such fragments. A sandwich assay, based on two monoclonal antibodies directed against human platelet glycoprotein IIb-IIIa complex (GP IIb-IIIa), enabled us to detect this complex in morphologically unidentifiable cell fragments. We consider GP IIb-IIIa a reliable marker for platelet-derived material, because these glycoproteins are almost exclusively present on platelets (George *et al*, 1984; Howard *et al*, 1982).

With this assay, the platelet character of platelet fragments made *in vitro* was shown as well as the occurrence of platelet-derived fragments in the blood of thrombocytopenic patients, probably produced by *in-vivo* platelet destruction.

MATERIALS AND METHODS

Isolation of platelets

Blood from patients and donors was collected in 10-ml tubes containing 4 mM EDTA (Venoject). Platelet-rich plasma (PRP) was obtained by centrifugation of the blood for 10 min at 350 g.

Sonication of platelets

Samples of 1 ml PRP were sonicated in 1-ml reaction tubes (Eppendorf), by using an MSE sonicator type 'Cabinet' no. 7100 with a probe (tip diameter 3 mm) at an amplitude of 8 microns peak to peak. A 15-sec period of sonication resulted in a disrupted platelet population as observed by a Coulter Channelyzer and by light microscopy.

Monoclonal antibodies

C17 (CLB-thrombo/1) has been described by Tetteroo *et al* (1983). This monoclonal antibody precipitates the glycoprotein IIIa (and some IIb) from solubilized human platelets.

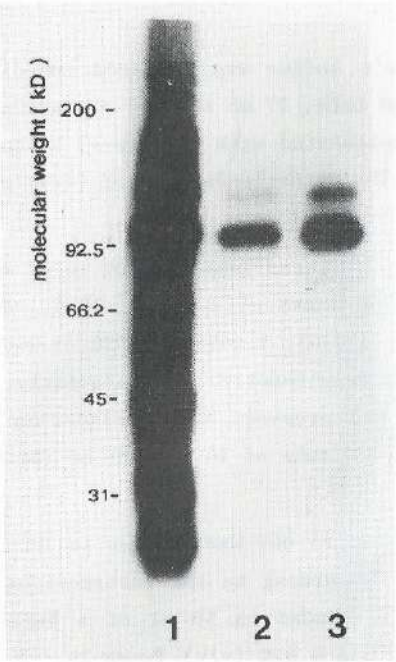

Fig. 1 - Autoradiogram of immune precipitates of C17 and 6C9 from ^{125}I-surface-labelled platelets of a normal healthy donor separated on a SDS-polyacrylamide gel [5-15% (w/v)]. Neither monoclonal antibody precipitated a band out of a lysate prepared from ^{125}I-labelled platelets of a patient with Glanzmann's thrombasthenia. Lane 1, total platelet lysate; lane 2, precipitation with McAb C17; lane 3, precipitation with McAb 6C9.

6C9 (CLB-thrombo/7) directed against GP IIb-IIIa was produced by M.J.E. Bos in our laboratory (unpublished). In serological studies, 6C9 appeared to react with human platelets, but not with platelets from patients with Glanzmann's thrombasthenia or with other human blood cells. Reactivity with the GP IIb-IIIa complex was observed after immunoprecipitation and SDS polyacrylamide gel electrophoresis and autoradiography (Fig. 1) (Modderman *et al*, in preparation). Both monoclonal antibodies were purified by chromatography on DEAE-Sephacel (Pharmacia) and their purity was confirmed by SDS-PAGE (Laemmli, 1970). Subsequently, they were iodinated by the Io-

dogen method (Fraker & Speck, 1980) and applied to an ACA-44 column to remove unbound ^{125}I and unreactive fragments of iodinated IgG. Peak fractions, reacting avidly with human platelets, were collected and stored at -20°C until use.

Tyrode's buffer

Calcium-free tyrode's buffer was prepared immediately before use and was composed of 130 mM NaCl, 27 mM KCl, 16 mM $NaHCO_3$, 0.36 mM $Na_2 HPO_4$, 5 mM $MgCl_2$, pH 6.8, supplemented with 0.2% (w/v) bovine serum albumin (BSA, Organon Teknika, Oss, The Netherlands) and 1% (w/v) glucose.

Albumin gradient

Bovine serum albumin (Organon Teknika) 30% (w/v) was diluted with tyrode's buffer to yield solutions of 7.5, 10, 12.5, 15, 17.5 and 20% (w/v) BSA (densities 1.0274, 1.0365, 1.0458, 1.0550, 1.0648 and 1.0745, respectively, as assessed by densitometry). Subsequently, a discontinuous six-step albumin gradient was prepared by underlayering 1 ml of each albumin solution in a poly-allomer tube of 14 mm x 89 mm (Beckman 331372).

Antibody coupling

One mg of purified C17 or 6C9 was coupled to 100 mg CNBr activated Sepharose 4B (Pharmacia) according to the instructions of the manufacturer. Sepharose beads were suspended in 50 ml of a buffer containing 0.05 M Tris-HCl, 0.15 M NaCl and 0.05% (v/v) Tween-20, pH 8.0 (TNT-buffer) and 0.01% (w/v) NaN_3.

Separation of platelets

In a typical experiment, PRP, diluted with one part of tyrode's medium (total volume 1-2 ml, containing 0.5-1.0x10^8 platelets) was carefully layered on top of the albumin gradient and centrifuged for 20 min at 800 g at room temperature. Fractions of 300 µl were collected. The number of platelets and/or platelet fragments present in each separate fraction and their size distribution curve were determined in a Coulter counter, model ZF, with a Channelyzer.

Radioimmunoassay for detection of glycoprotein IIb-IIIa complex (RIA for GP IIb-IIIa)

GP IIb-IIIa was used as a marker for platelets and platelet fragments. To yield GP IIb-IIIa complexes, platelets and/or platelet fragments were solubilized with Nonidet P40 (NP40, Sigma).

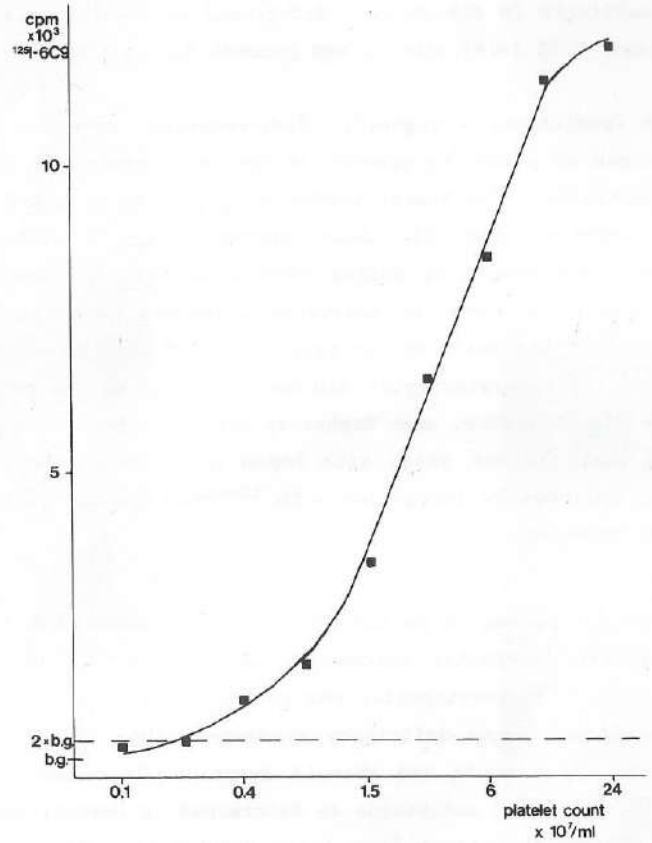

Fig. 2 - Dose-response curves of a twofold dilution series of normal human platelets in the RIA for GP IIb-IIIa.

In a typical experiment, 300 µl of a sample containing platelets and/or platelet fragments, were incubated with 300 µl TNT buffer containing 1% (v/v) NP40, 1% (w/v) albumin and 0.25 mg Sepharose beads coupled with 2.5 µg purified C17. After a 16-h incubation, the beads were washed 4 times with 0.9% (w/v) NaCl solution by centrifugation at 2000 g and subsequently incubated with ^{125}I-labelled C17 or 6C9. The Sepharose beads were then washed again 4 times with 0.9% (w/v) NaCl solution and the amount of radioactivity bound to the beads was measured in a gamma counter. GP IIb-IIIa was only bound when C17 was used as the antibody coupled to Sepharose beads and, when radiolabelled, 6C9 was used to complete the sandwich.

Furthermore, a final concentration of 0.5% (v/v) NP40 appeared to be essential for reactivity in the assay. Background radioactivity was below 1.0% when at least 0.5% (w/v) albumin was present during the first incubation.

Under these conditions, a sigmoidal dose-response curve was obtained between the number of platelets present in the test sample and the amount of bound radioactivity. The lowest number of platelets detected in this way was about $4x10^6$/ml (Fig. 2). Dose-response curves constructed with platelets solubilized before or during incubation with C17-Sepharose as well as a dose-response curve of sonicated platelets were similar. As could be expected on the basis of the specificity of both monoclonals, an excess of leukocytes or erythrocytes did not interfere with a proper binding of GP IIb-IIIa. Moreover, when Sepharose beads, coupled with monoclonal antibodies that did not react with human platelets, were incubated with platelets, followed by incubation with ^{125}I-6C9, no specific binding of ^{125}I-6C9 was observed.

Patients

Five patients are presented in this study: two patients (A and B) suffering from chronic idiopathic thrombocytopenia (ITP), one patient (C) from a drug-induced thrombocytopenia, one patient from a thrombocytopenia accompanying acquired immune deficiency syndrome (AIDS) (D) and one patient from a thrombocytopenia and chronic lymphatic leukaemia (E). All patients had platelet-bound antibodies as determined by immunofluorescence (Borne *et al*, 1980). In patient A, a large quantitative discrepancy was found between the results in this test (weakly positive reaction with anti-IgG) and the results in a simultaneously performed consumptive radioimmunoassay for the measurement of platelet-associated IgG (PA-IgG, 16.1 fg-IgG per platelet, normal donors <0.8 fg IgG per platelet) (Vos *et al*, submitted for publication).

RESULTS

Size distribution curves of platelets on an albumin gradient

As apparent from representative data, as shown in Fig. 3, this method is a convenient and highly reproducible method for the separation of platelets of different sizes. Separation of platelets by this protocol occurs not only because of differences in density, but also because of differences in size. In each fraction, the number of particles was determined as well as their size. Platelets from normal donors appeared to be distributed over the gradient in a log-normal manner (Fig. 4). Most of the platelets appeared in the 15 and 17.5% (w/v) albumin-density area. Modal platelet size declined in parallel with decreasing albumin density.

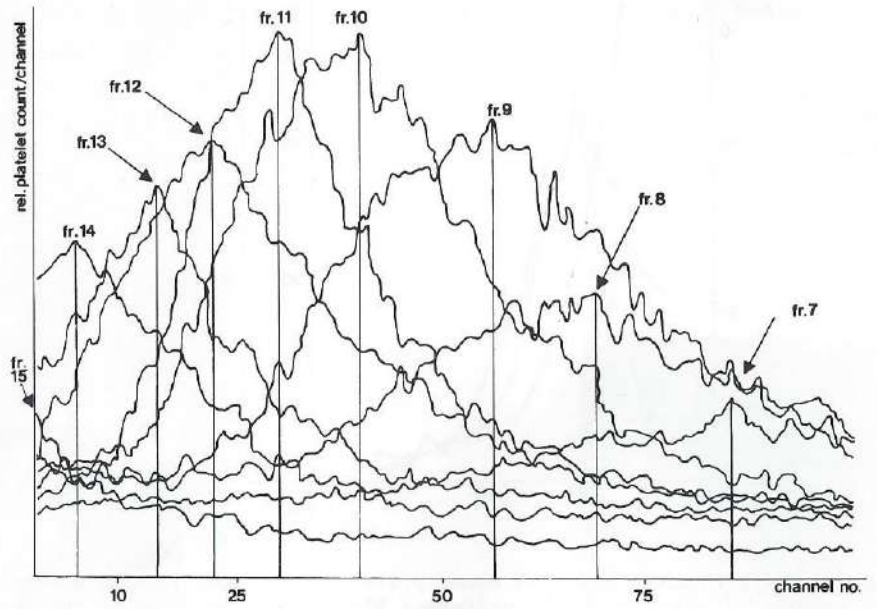

Fig. 3 - Size distribution plots detected by Coulter Channelyzer of various fractions collected from a discontinuous albumin gradient. Modal platelet size decreases as albumin density decreases.

When one-half of the PRP was sonicated and subsequently added to the (unsonicated) half of the PRP, a size-distribution curve with two peaks was found (Fig. 5A). The peak in the 15-17.5% (w/v)-density area, representing intact platelets, was significantly smaller whereas a new peak appeared in the 7.5-10% (w/v) albumin and plasma area, obviously containing particles of smaller size. Moreover, when ^{125}I-labelled platelets were sonicated, most radioactivity was measured in the 7.5-10% (w/v) albumin and plasma area (data not shown). A re-run of a fraction of particular density, such as from the 7.5-12.5% (w/v) area, resulted in the same size distribution range of that particular area (Fig. 5B).

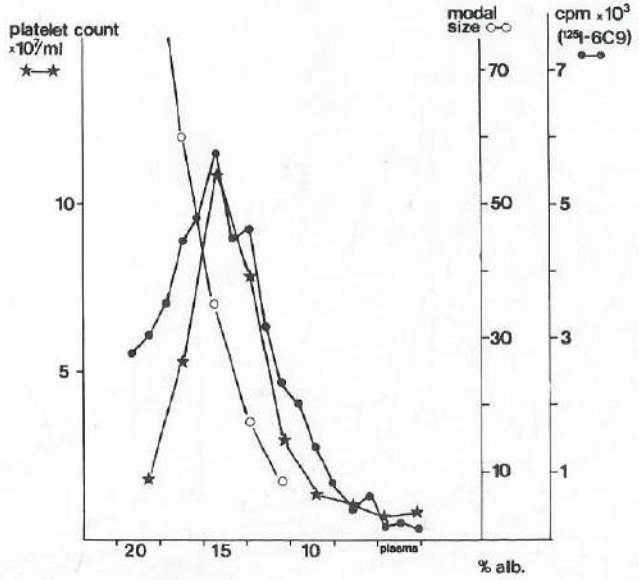

Fig. 4 - Distribution of normal donor platelets over the albumin gradient, and results of simultaneously performed RIA for GP IIb-IIIa on the separate fractions. Platelets sedimented mainly in the 15-17.5% (w/v)-density area.

RIA for GP IIb-IIIa of various albumin gradient fractions

A plot of the values obtained from the RIA for GP IIb-IIIa when applied on different albumin gradient fractions, paralleled the curves of the number of particles determined by the Coulter counter (Figs. 4 and 5). Thus, particles derived from platelet sonication could be assessed by our assay as being of platelet origin. Note that GP IIb-IIIa complexes were also detected in the plasma area of the gradient.

Investigations on PRP from patients

In the PRP of all 5 patients, particles smaller than normal platelets were detected (Fig. 6). These particles appeared in the 7.5-10% (w/v) albumin area or remained in the plasma top fraction. Interestingly, in fractions in which the number of platelets was too low to be detectable by the Coulter counter, GP IIb-IIIa was still detected and even distributed in a log-normal manner. In patient A (platelet count $10 \times 10^9/l$), platelet material was observed mainly in the 7.5-10% (w/v) albumin and plasma fractions, the normal platelet peak being virtually absent. However, the re-

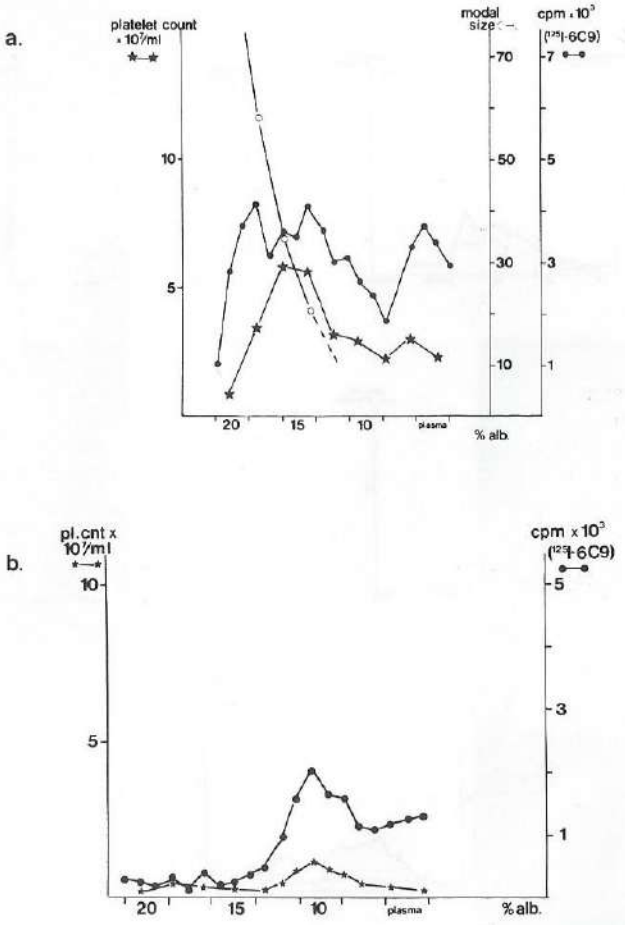

Fig. 5A - Distribution of 0.5 ml of sonicated PRP from a normal donor added to 0.5 ml normal donor PRP, and results of a simultaneously performed RIA for GP IIb-IIIa on the separate fractions. Compared with the distribution in Fig. 4, the peak of normal platelets in the 15-17.5% (w/v)-density area was smaller, whereas a new peak appeared in the 7.5-10% (w/v) albumin and plasma area. Note that the curves of the counted particles and of the results in the RIA for GP IIb-IIIa parallel each other, whereas the modal platelet-size distribution over the gradient remains the same.
Fig. 5B - Results of a re-run of a fraction of the 7.5-12.5% (w/v)-density area on a new albumin gradient and the simultaneously performed RIA for GP IIb-IIIa.

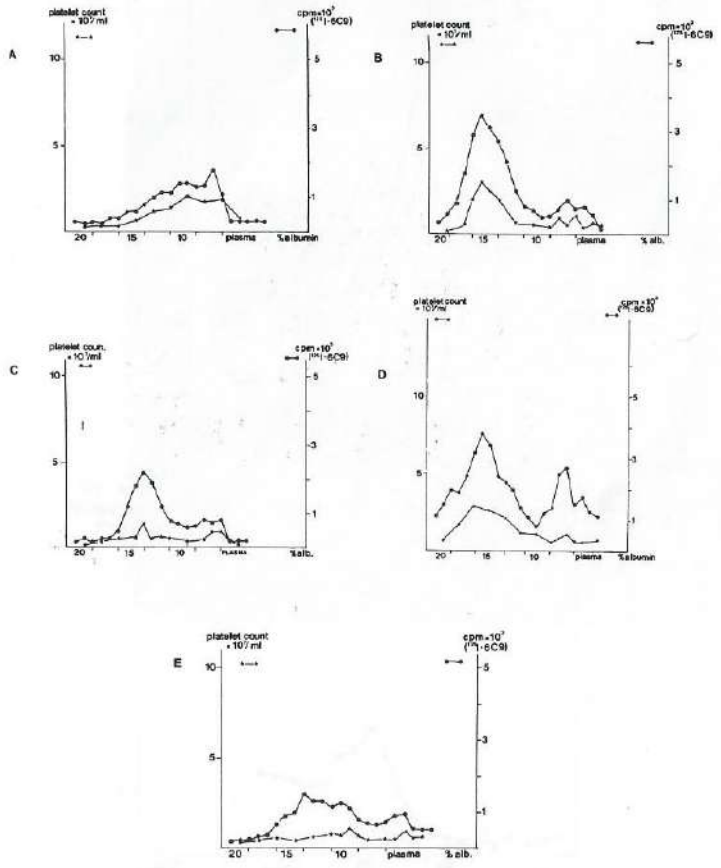

Fig. 6 - Distribution of PRP of five thrombocytopenic patients (A-E) over the albumin gradient, and results of a simultaneously performed RIA for GP IIb-IIIa on the separate fractions. The modal platelet-size distribution over the gradient, as far as could be determined by Coulter Channelyzer, was similar to that of normal donor PRP (data not shown).

sults of the RIA for GP IIb-IIIa completely paralleled the curve of the particles detected by the Coulter counter, which makes it likely that these particles were indeed of platelet origin.

DISCUSSION

The availability of 2 different monoclonal antibodies directed against the platelet-specific glycoprotein IIb-IIIa complex (GP IIb-IIIa) enabled us to develop a radioimmunoassay to detect particles containing this GP complex. The two monoclonal antibodies are probably directed against a conformational antigenic determinant on the GP IIb-IIIa complex, because both can precipitate GP IIb-IIIa from solubilized platelets, and they do not react with the separate constituents of the complex in the immunoblotting technique, i.e. after SDS-polyacrylamide gel electrophoresis and subsequent electrotransfer of the proteins to nitrocellulose (results to be published). Moreover, their reactivity in the RIA depended on the continuous presence of 0.5% (v/v) Nonidet P40, which also indicates that a conformation close to that of the native protein complex is necessary. In the sandwich RIA that we developed, it appeared that GP IIb-IIIa could only be detected when the two monoclonal antibodies were applied sequentially and only when one (C17) was bound to the Sepharose beads and the other (6C9) was used as the radiolabelled probe for the detection of GP IIb-IIIa binding. Why these antibodies can only be used in this particular combination and not vice versa is not clear yet.

According to Coulter counter measurements, the albumin gradient that we applied separated intact platelets effectively from fragmented ones. Loss of fragmented platelets was avoided by layering of PRP on top of the albumin gradient and by testing each fraction in the RIA without further processing. This assay made it possible to determine the platelet nature of particles smaller than normal platelets, not only artificially prepared by sonication, but also circulating in the blood of thrombocytopenic patients.

This proves the platelet nature of the circulating fragments present in such disease states due to increased platelet destruction (Kahn *et al*, 1975; Zucker-Francklin & Karpatkin, 1977; George *et al*, 1982).

As mentioned before, occurrence of platelet fragmentation is considered to be one of the causes of aspecifically elevated PA-IgG values or unexpectedly high PA-IgG values which may occur in such patients (Shulman *et*

al, 1982). Platelet fragments may contribute to the amount of IgG in a test suspension, whereas they cannot be accurately counted. This is illustrated by the results obtained in one patient (A; Fig. 6), in whom we found a dubious reaction with anti-IgG in the immunofluorescence test (in which only intact platelets are examined) together with a strongly elevated PA-IgG. This patient had virtually no normally sized platelets in his blood, as observed by albumin gradient-size separation and the RIA for GP IIb-IIIa, but high amounts of fragmented platelets.

In summary, we developed a RIA for platelet IIb-IIIa which could be used to measure platelets as well as platelet-derived material. Together with an albumin-gradient method for the separation of intact or fragmentated platelets of different sizes, this method makes it possible to study platelet fragmentation *in vitro* as well as *in vivo*, i.e. in patients suffering from diseases with increased destruction of platelets.

REFERENCES

BORNE, A.E.G. Kr. von dem, HELMERHORST, F.M., LEEUWEN, E.P. van, PEGELS, J.G., Von, RIESZ, E. & ENGELFRIET, C.P. (1980) Autoimmune thrombocytopenia: detection of platelet autoantibodies with the suspension immunofluorescence test. *British Journal of Haematology*, 45, 319-327.

FRAKER, P.J. & SPECK, J.C. (1980) Protein and cell membrane iodinations with sparingly soluble chloramide-1,2,4,6-tetra chloro-3α,6α-diphenyl glycosil. *Biochemical and Biophysical Research Communications*, 4, 849-857.

GEORGE, J.N., NURDEN, A.T. & PHILLIPS, D.R. (1984) Molecular defects in interactions of platelets with the vessel wall. *New England Journal of Medicine*, 311, 1084-1089.

GEORGE, J.N., THOI, L.L., McMANUS, L.M. & REIMAN, T.A. (1982) Isolation of human platelet membrane particles from plasma and serum of patients with ITP. *Blood*, 60, 834-840.

HOWARD, L., SHULMAN, S., SADAMANSAN, S. & KARPATKIN S. (1982) Crossed immunoelectrophoresis of human platelet membranes. The major antigen consists of a complex of glycoproteins, GPIIb and GPIIIa, held together by Ca^{2+} and missing in Glanzmann's thrombosthenia. *Journal of Biological Chemistry*, 257, 8331-8336.

KHAN, I., ZUCKER-FRANKLIN, D. & KARPATKIN, S. (1975) Microthrombocytosis and platelet fragmentation associated with idiopathic/autoimmune thrombocytopenia. *British Journal of Haematology*, 31, 449-460.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

SHULMAN, N.R., LEISINGER, C.A., HOTCHKISS, A.J. & KANTZ, C.A. (1982) The non-specific nature of platelet-associated IgG. In: *Transactions of the Association of American Physicians*, Vol.XCV 213-220.

TETTEROO, P.A.T, LANSDORP, P.M., LEEKSMA, O.C. & BORNE, A.E.G. Kr von dem (1983) Monoclonal antibodies against human platelet glycoprotein IIIa. *British Journal of Haematology*, 55, 509-522.

ZUCKER-FRANCKLIN, D. & KARPATKIN, S. (1977) Red cell and platelet fragmentation in idiopathic autoimmune thrombocytopenic purpura. *New England Journal of Medicine*, 297, 517-523.

CHAPTER VI

MONITORING OF PLATELET CONTAMINATION IN FILTERED RED BLOOD-CELL CONCENTRATES

Use of a radioimmunoassay detecting the platelet glycoprotein IIb-IIIa complex

J.J.E. Vos, C. Schoen, H.K. Prins, A.E.G. Kr. von dem Borne and J.G. Huisman

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

ABSTRACT

Monitoring of contaminating platelets, granulocytes and lymphocytes in leukocyte-poor red blood-cell concentrates is usually done by counting in a electronic particle counter. The sensitivity and the specificity of this technique are compromized by the contamination of the preparations with other cell types and particles thereof.

In this report, a sensitive and specific radioimmunoassay (RIA) detecting the platelet glycoprotein IIb-IIIa complex (GP IIb-IIIa) is described.

Our results indicate that platelet fragmentation, not detectable for particle counters, may occur when blood-cell suspensions are filtered. Therefore, even when no intact platelets pass through the filter, platelet antigenic material may find its way in the red blood-cell concentrate. This finding might have important implications for the preparation of leukocyte-free red blood cells. Unwanted immunization might occur after transfusion, especially when not only platelets but also leukocytes would be fragmented by procedures by which the blood is made leukocyte-poor.

Submitted for publication

INTRODUCTION

Red blood-cell concentrates can be freed from leukocytes and platelets by filtration through cotton wool (1). Filtered red blood-cell concentrates are used to avoid undesired alloimmunization by contaminating leukocytes and/or platelets as well as graft-versus-host reactions after red blood-cell transfusion.

Usually, the efficiency of a filtration procedure is checked by counting the number of contaminating platelets and/or leukocytes in an electronic particle counter. Counting of leukocytes can be done directly and indirectly in saponin-treated blood samples. In contrast, platelets must be separated from other blood cells before the number of contaminating platelets can be determined by the electronic particle counter. Unfortunately, remaining red blood cells interfere with proper determination of the number of platelets. Moreover, cell fragments of platelets or leukocytes, if present in the test samples, may not be counted by the electronic particle counter.

Recently, we developed a radioimmunoassay for the detection of platelets and platelet fragments, using two different monoclonal antibodies directed against the human platelet glycoprotein IIb-IIIa complex (GP IIb-IIIa) (2,3).

The aim of this study was to compare the results obtained with this radioimmunoassay and the results of particle counting measurements in monitoring platelet (or platelet fragments) contamination in filtered red blood-cell concentrates.

MATERIALS AND METHODS

Radioimmunoassay for the detection of glycoprotein IIb-IIIa complex (RIA for GP IIb-IIIa)

This method has been developed recently (3). Two monoclonal antibodies (McAb; C17 = CLB thrombo 1 and 6C9 = CLB thrombo 7, both purified by DEAE-Sephacel column chromatography), directed against the platelet-specific GP IIb-IIIa (2,3) were used to detect platelets and/or platelet-derived material. In short, blood samples were solubilized with Nonidet P40 (NP40, Sigma Chem. Co., St. Louis, MO, USA) to yield GP IIb-IIIa complexes. In a typical experiment, 300 µl of a (blood)sample, possibly containing platelets and/or platelet-derived material, were incubated with 300 µl of a buffer containing 0.05 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20, pH 8.0 (TNT buffer), 1% NP40 (v/v) and 2.5 µg C17 McAb, coupled to 0.25 mg Sepharose beads (Sepharose CNBr, Pharmacia Fine Chemicals, Uppsala, Sweden).

After a 16-h incubation period, the beads were washed four times with 0.15 M NaCl solution by resuspension and centrifugation up to 2000 g and subsequently incubated with ^{125}I-labelled 6C9 McAb for 6 h in TNT buffer containing 1% NP40 (v/v), 0.6% human IgG (w/v) and 3% bovine serum albumin (w/v). The Sepharose beads were then washed again four times with 0.15 M NaCl solution and the number of radioactivity bound to the beads was measured in a gammacounter. Using the assay, a sigmoidal dose-response curve could be obtained between the number of platelets present in a test sample and the amount of bound ^{125}I-labelled 6C9 McAb to the coupled GP IIb-IIIa complex with a wide linear range between platelet counts from 8 - 120x10^6/ml. The lowest number of platelets detected was about 4x10^6/ml (see Chapter V, Fig. 2). In a control experiment, dose-response curves of washed platelets in the presence of 5x10^8 erythrocytes/ml parallelled similar curves without erythrocytes.

Filtration of red blood-cell concentrates

For this purpose, we used Cellselect® leukocyte filters (NPBI, Emmer Compascuum, The Netherlands) which have a capacity to free one unit of red blood-cell concentrate of virtually all their leukocytes and platelets. In this study, we deliberately overloaded the Cellselect leukocyte filter to ensure that platelets and/or platelet-derived material would pass the filter procedure. Therefore, three units of red blood-cell concentrates, each diluted with an equal volume of 0.15 M NaCl were filtered over a Cellselect leukocyte filter that had been prewashed with 0.15 M NaCl. The filtrate was collected in fractions of 100 ml. Aliquots (vol. 100 µl diluted to 300 µl with TNT buffer) were tested in triplicate for their content of platelets and/or platelet-derived material. Simultaneously, the number of platelet-sized particles in each fraction was determined by a Coulter electronic particle counter, model ZF (attenuation 0.5, aperture 4, threshold 10). For each sample, the Coulter counts were corrected for erythrocyte content (attenuation 2, aperture 32, threshold 11). Also, the size distribution pattern of each sample was checked for the presence of a normal platelet peak by a Coulter Channelyzer.

Separation by albumin-density gradient

In filtration procedure II, samples of 1 ml of fractions no. 1 and 10 were layered on a 7.5-20% (w/v) albumin-density gradient and centrifuged for 20 min at 800g, as described before (3), to separate normal sized platelets from fragmented platelets. Subsequently, fractions of 300 µl were collected from each gradient and tested for their GP IIb-IIIa content. Also, in each fraction, the number of platelet-sized particles and their size distribution curve was determined in the Coulter counter ZF (aperture 0.5, attenuation 4, threshold 10) with Channelyzer.

RESULTS

In Fig. 1, the results of two filtration procedures are shown. In the various fractions, collected after filtration, virtually no platelet-sized particles were counted by the electronic particle counter. Also, no normal

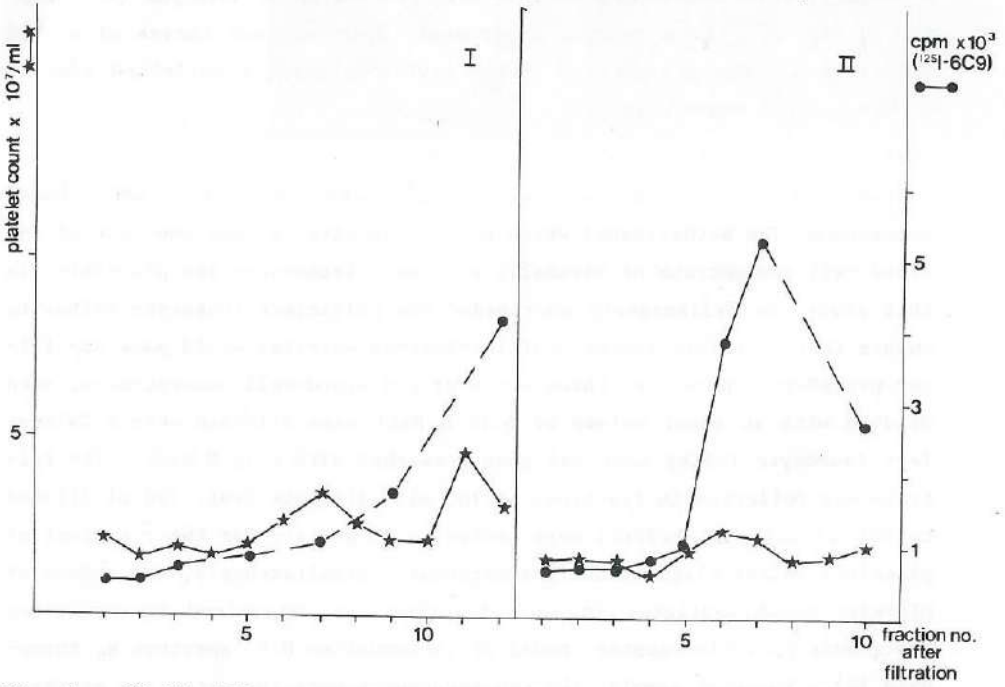

Fig. 1 - GP IIb-IIIa measurement in fractions collected after filtration of an overload (three units) of packed cells over a blood-cell filtration column. Gradually, more GP IIb-IIIa was detected in subsequent fractions, whereas platelet counts in separate fractions practically did not differ from each other.

platelet-size distribution curves were observed by particle channelyzing. In contrast, in both filtrations, gradually more GP IIb-IIIa complex was detected by the RIA upon overload of the filter.

In Fig. 2, results of RIA for GP IIb-IIIa, applied to the separate fractions collected after albumin-density separation of samples taken from fractions nos. 1 and 10 of filtration no. II, are shown. In fraction no. 1, hardly any GP IIb-IIIa complex could be detected. In contrast, in fraction no. 10 of filtration II, GP IIb-IIIa complexes were detected in the 7.5% area of the albumin gradient and the 'plasma' area, but not in the

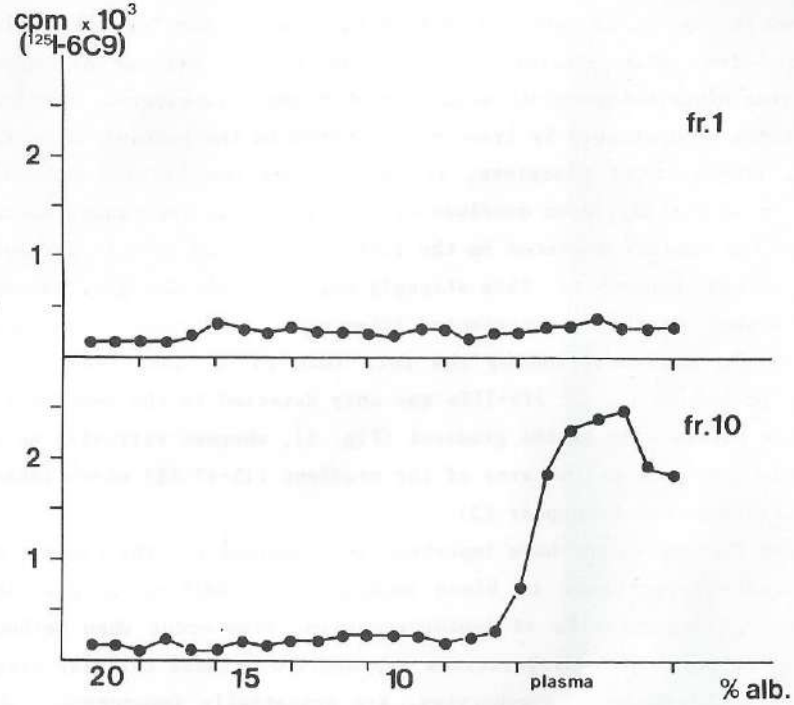

Fig. 2 - GP IIb-IIIa measurement on fractions collected after albumin-gradient separation of 1 ml of fractions nos. 1 and 10 collected of blood-cell filtration procedure II: GP IIb-IIIa was detected only in the 7.5% area and 'plasma' area of filtration fraction no. 10, whereas in filtration fraction no. 1 virtually no GP IIb-IIIa was detected.

15-17.5% area of the albumin gradient, where normal intact platelets are expected to be present.

DISCUSSION

In this study, we have demonstrated an application of a radioimmunoassay detecting GP IIb-IIIa in blood banking. With this technique, it appeared to be possible to detect contamination of filtered whole blood with platelets and/or platelet fragments, whereas the technique usually employed to detect contaminating platelets (an electronic particle counter measurement) proved inadequate. The advantage of the RIA for GP IIb-IIIa in this respect is that it is a very sensitive technique for detecting both intact platelets and their fragments.

As shown in Fig. 2, it appeared that in this particular filtration procedure very few intact platelets passed the filter, because no normal platelet-size distribution plots were seen with the channelyzer. Particle counting might be disturbed by lysed erythrocytes in the samples or by the fact that fragments of platelets, if they passed the filter, were not counted. In this study, upon overload of the filter, an increasing amount of GP IIb-IIIa complex appeared in the filtrate while no intact platelets were seen on the channelyzer. This strongly suggests that GP IIb-IIIa complex that passed the filter represented fragmentated platelets. This suggestion was further supported by the fact that in the last fraction of filtration procedure II, GP IIb-IIIa was only detected in the low-density area and the plasma area of the gradient (Fig. 2), whereas virtually no GP IIb-IIIa was detected in the area of the gradient (15-17.5%) where intact platelets are expected to appear (3).

The above findings might have important implications for the control of leukocyte-poor preparations in blood banking. In addition to platelet fragmentation, fragmentation of leukocytes might also occur when methods to produce leukocyte-poor preparations are applied. These cellular fragments, especially those of lymphocytes, are potentially immunogenic substances. A sensitive test, such as the RIA for GP IIb-IIIa, detecting intact and fragmentated platelets, as well as similar tests that have been developed for lymphocyte- and granulocyte-derived material (Schoen et al, in preparation), might be tools in screening for immunogenic substances, both in leukocyte-poor red blood-cell concentrates before transfusion and in the quality control of procedures used for preparation of leukocyte-free red blood-cell concentrates.

REFERENCES

1. Diepenhorst, P.; Sprokholt, R.; Prins, H.K.: Removal of leucocytes from whole blood and erythrocyte suspensions by filtration through cotton wool. I. Filtration technique. Vox Sang *23*: 308 (1972).

2. Tetteroo, P.A.T.; Lansdorp, P.M.; Leeksma, O.C.; Von dem Borne, A.E.G. Kr. Monoclonal antibodies against human platelet glycoprotein IIIa. Brit. J. Haematol. *55*: 509 (1983).

3. Vos, J.J.E.; Huisman, J.G.; Modderman, P.W.; Von dem Borne, A.E.G. Kr. Detection of circulating human platelet fragments using monoclonal antibodies against platelet glycoprotein IIb-IIIa complex. Submitted for publication.

CHAPTER VII

TARGET ANTIGENS FOR PLATELET-SPECIFIC AUTO- AND ALLOANTIBODIES. IDENTIFICATION BY IMMUNOBLOTTING TECHNIQUE

J.J.E. Vos, A.E.G. Kr. von dem Borne and J.G. Huisman

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands

SUMMARY

In this study, preliminary results are presented of an immunoblot analysis of sera containing platelet autoantibodies, alloantibodies or antibodies directed against so-called cryptantigens (EDTA- or PFA-dependent antibodies) and platelet eluates.

Immunoblot analysis proved to be helpful in determining the specificity of a serologically ambiguously reacting alloantiserum. In contrast, only 2 out of 24 sera, obtained from patients suffering from idiopathic thrombocytopenic purpura (ITP), reacted with specific bands, namely one serum with a 130-kD band and a 240-kD band, and one serum with a 240-kD band only. Reactivity with this 240-kD band was also observed with the 4 sera containing antibodies against cryptantigens. Furthermore, none of the 12 tested eluates prepared from platelets of patients with ITP reacted with specific bands.

The lack of reactivity of platelet autoantibodies containing sera and eluates in immunoblots might be explained by the loss of (conformational) determinants of the target antigens during the preparation of the platelet suspension, solubilization and/or electrophoresis.

Furthermore, our results indicate that circumstances before or during the immunoblot procedure, such as activation of platelets by low temperatures and addition of EDTA to the solubilization mixture for the inhibition of protease activity, may influence the reactivity of sera in the immunoblot technique.

INTRODUCTION

In the past few years, radioimmunoassays using purified platelet glycoproteins, radioimmunoprecipitation and immunoblotting have been employed for the identification of target antigens for platelet-specific auto- and alloantibodies (1-10).

In the case of alloantibodies, the immunoblotting technique has led to the localization of the Zw system (= PI^A) on the platelet glycoprotein IIIa (2,4) and of the platelet-specific antigens Bak^a (4) and Lek^a (10) on the platelet glycoprotein IIb.

Localization of target antigens for platelet autoantibodies has been more difficult. In the vast majority of sera tested in immunoblot, no reactivity has been observed, whereas in a small number of sera reactivity with the platelet glycoprotein IIIa and a protein with a relative molecular weight of 200-250 kD has been observed in the immunoblotting technique (5,7,9).

The aim of this study was to extend previous observations done in our laboratory (4) with the immunoblotting technique. Platelet-specific auto- and alloantisera were tested, as well as ether eluates prepared from platelets of patients with ITP, with a high reactivity with donor platelets in the platelet immunofluorescence test (PIFT). Also, the reactivity of sera, containing so-called EDTA- and PFA-dependent antibodies (11,12), was tested.

MATERIALS AND METHODS

Platelet preparation

Blood was collected from phenotyped healthy volunteers and from a patient with Glanzmann's disease type I. Platelet preparation was essentially as described before (13).

Sera

Anti-Zw^a and anti-Bak^a sera were obtained from women immunized during pregnancy or from patients who had suffered from post-transfusion purpura.

The anti-Lek^a serum was a kind gift from Dr. Boizard and has been described in detail (14). Twenty four sera, obtained from patients fulfilling the diagnostic criteria for idiopathic thrombocytopenia (see Chapter I), were tested. Each serum reacted positively in the PIFT (13). Serum Ve, obtained from a patient with pseudo-thrombocytopenia, contained antibodies reacting in the PIFT only in the presence of EDTA (so-called EDTA-dependent antibodies) (11,12). Sera Ko and Zw were obtained from patients with idiopathic thrombocytopenia and contained antibodies reacting in the PIFT with paraformaldehyde (PFA)-fixed platelets but not with unfixed donor platelets (so-called PFA-dependent antibodies) (11,12). Also, 12 eluates prepared from the platelets of ITP patients, strongly reactive in the PIFT (score 3+ or 4+), were examined. Two of these eluates reacted with Glanzmann type-I platelets.

Radiolabeled anti-IgG

Murine monoclonal anti-human IgG (CLB no. M1268 MH16M) or $F(ab')_2$ rabbit-anti-human IgG (CLB no. M1023 KH16P) was used for the detection of specifically bound antibodies. Both preparations were iodinated according to the Iodogen method (15).

Platelet solubilization and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Platelets (10^9) were allowed to solubilize at room temperature for 45 min in 200 µl of a solubilization mixture containing 10 mM Tris-HCl, pH 8.0, 1% (v/v) Nonidet P40 (NP40), 5 mM N-ethylmaleamide (NEM), 5 mM EDTA, 2 mM PMSF and trypsin inhibitor (20 µg/ml). In some experiments, NEM or EDTA was omitted from this mixture. The lysate was cleared from cell debris by centrifugation at 12 000 g for 30 min at 4°C. The supernatant was then incubated for 30 min at room temperature with 1 mg swollen protein-A Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden)/ml lysate to remove traces of platelet-associated IgG. Finally, 125 mM Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulfate (SDS, w/v) and 20% glycerol (v/v) were added to the cleared lysate in a ratio of 1:4 and the mixture was boiled for 5 min. SDS-PAGE was carried out according to Laemmli (16).

Immunoblotting

Immunoblotting was performed as described (6) with slight modifications. After electrophoretic transfer, the nitrocellulose sheet was washed to remove loosely bound material. The remaining active sites were blocked in a solution containing 1% (w/v) gelatin and 0.5% (v/v) NP40 in PBS for 2 h at 37°C. It was then incubated for 30 min at 37°C in PBS to remove excessive gelatin deposits. Subsequently, the nitrocellulose sheet was mounted in a serum incubator, developed in our laboratory (A.A.A. Westgeest, unpublish-

ed), allowing 21 serum incubations at a time.

Sera and eluates, diluted 1:20 (eluates 1:5 and 1:10) in PBS containing 0.1% (v/v) Tween-20 and 0.2% (w/v) gelatin, were incubated in a volume of 1 ml in this incubator at room temperature. After 2 h, the nitrocellulose paper was rinsed twice during 1 h in 250 ml of washing buffer, containing 0.5% (v/v) Tween-20 and 0.2% (w/v) gelatin in PBS. It was then incubated for 30 min with 100 ml PBS with 0.1% (v/v) Tween-20 and 0.2% (w/v) gelatin containing 5 x 10^6 cpm of ^{125}I-labeled anti-human IgG antibodies. Subsequently, it was rinsed again twice for 1 h in washing buffer. Finally, the sheet was exposed to Kodak X-Omat-XAR film using a Dupont Cronex lighting Plus AH-intensifying screen at -70°C.

RESULTS

Immunoblot analysis with alloantisera

Fig. 1 shows a representative example of the immunoblot analysis to establish the reactivity of platelet alloantibodies anti-Zw^a and anti-Bak^a with platelet solubilizates of Zw(a+) and Bak(a+) donors. As is apparent from lane a, anti-Zw^a reacted with a 88-kD protein, representing the GP IIIa molecule. Eluates prepared from Zw(a+) donor platelets, sensitized with anti-Zw^a sera, reacted also with the 88-kD band. The sensitivity of the immunoblot analysis to monitor antibody to GP IIIa in ether eluates appeared to be of the same order as the PIFT .

Anti-Bak^a and anti-Lek^a sera reacted with a 130-kD band on protein blots prepared from Bak(a+) and Lek(a+) donor platelets. This band represents the GP IIb molecule (Fig. 1). In contrast to the anti-Zw^a sera, sera containing anti-Bak^a antibodies showed reactivity with reduced GP IIb (120 kD).

An alloantiserum (Fa), reacting with both Zw(a+) and Zw(a-) donor platelets in the PIFT, reacted only with a 88-kD band (GP IIIa) on protein blots prepared with Zw(a+) donor platelets (Fig. 1).

Immunoblot analysis of other sera

Only two sera out of the 24 ITP sera tested reacted in immunoblot: sera Es and Ro. Furthermore, the sera containing EDTA- or PFA-dependent antibodies showed reactivity in immunoblot. Reactivity was observed with a 130-kD band (GP IIb) and a 240-kD band.

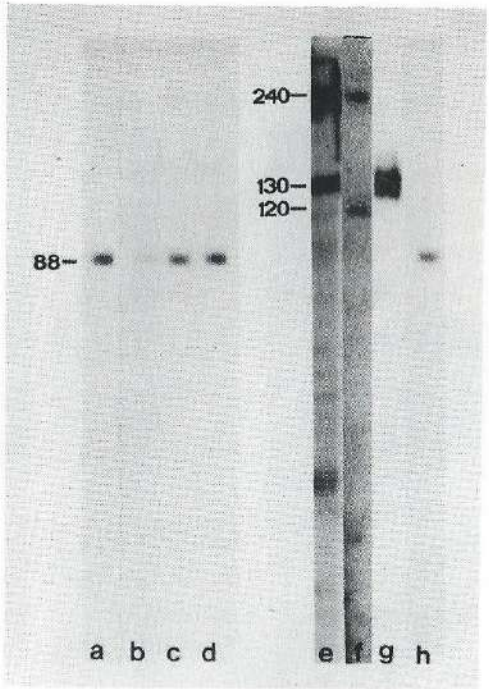

Fig. 1 - Immunoblots of various alloantisera, performed as described in Materials and Methods. All sera were diluted 1:20 unless stated otherwise. Lane a, anti-Zw^a serum Br. Lanes b, c and d, eluate of Zw(a+) platelets, sensitized with anti-Zw^a serum Br, diluted 1:20, 1:10 and 1:5, respectively. Lane e, anti-Bak^a serum Kl under non-reducing conditions. Lane f, as lane e, under reducing conditions. Lane g, anti-Lek^a serum, under non-reducing conditions. Lane h, serum Fa. Numbers refer to molecular weight (in kilo Daltons).

Reactivity with the 130-kD band (GP IIb)

The antibodies of patient Es reacted with protein bands with an apparent molecular weight of 130 kD and 240 kD on protein blots prepared from both Bak(a+) and Bak(a-) donor platelets (Fig. 2). This serum only reacted with the 240-kD band of a solubilizate prepared from Glanzmann type-I donor platelets. The reactivity of this serum with GP IIb remained upon reduction of GP IIb prior to the electrophoresis (cf. Fig. 1), whereas it increased when protein blots were prepared from platelets solubilized in the absence of 5 mM EDTA.

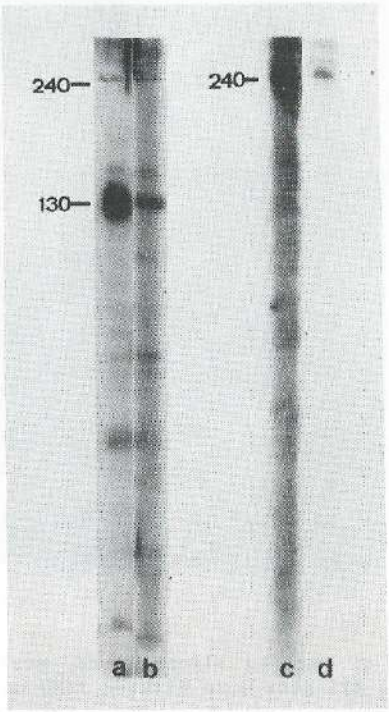

Fig. 2 - Immunoblots of autoantisera Es and Ro, performed as described in Materials and Methods. All sera were diluted 1:20 unless stated otherwise. Lane a, serum Es with solubilizate prepared in the absence of EDTA under non-reducing conditions. Lane b, as lane a, however, solubilizate prepared in the presence of 5 mM EDTA. Lane c, serum Ro, diluted 1:20. Lane d, as lane c, but diluted 1:100. Number refer to molecular weight (in kilo Daltons).

Reactivity of sera with unidentified platelet proteins

ITP serum Ro and dilutions thereof, sera with PFA-dependent antibodies (K and Zw) and a serum with EDTA-dependent antibodies (Ve), revealed reactivity with the 240-kD band which was also found in serum Es and anti-Bak^a sera. This reactivity was observed on protein blots prepared from donor platelets of any phenotype and platelets from the Glanzmann type-I patient.

The reactivity was lost when NEM was omitted from the solubilization mixture and diminished when platelets were activated by storage at 4°C during 60 min before solubilization. The effects of omitting NEM and platelet activation on the presence of the 240-kD band are shown in Fig. 3.

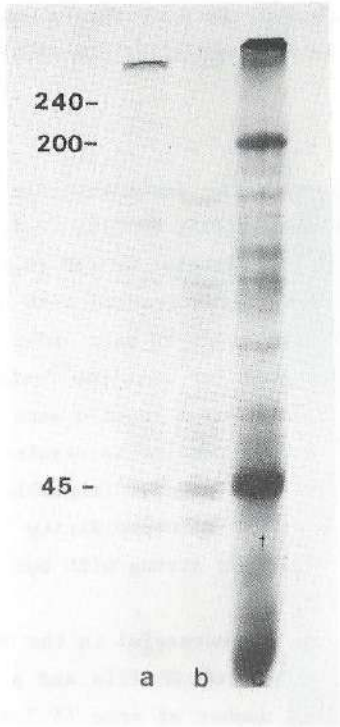

Fig. 3 - The 240-kD band observed after SDS-PAGE and stained with Coomassie Brilliant Blue. Lane a, solubilizate of Zw(a+)Bak(a+) platelets prepared in the absence of EDTA at 20°C. Lane b, as lane a, but after storage of the washed platelets at 4°C during 1 h and subsequent solubilization at 4°C. Lane c, as lane a, but without N-ethylmaleamide added to the solubilization mixture. Numbers refer to molecular weight (in kilo Daltons).

Immunoblot analysis with ether eluates from platelets of ITP patients

Twelve eluates prepared from PIFT-positive patient platelets (PIFT score: 3+/4+) were examined in the immunoblot procedure. Protein blots were prepared from Zw(a+), Bak(a+) donor platelets and from Glanzmann type-I platelets.

No reactivity in immunoblot was observed in any of the eluates, not even with a 1:5 dilution or when the autoradiography-exposition time was increased, although all had strong reactivity in the PIFT.

These eluates were applied to an immunoprecipitation technique with Triton X-100-solubilized ^{125}I-radiolabeled platelets obtained from Zw(a+), Bak(a+) donors. In this way, in 5 of them a specific binding to the GP IIb-IIIa complex could be observed after SDS-PAGE and autoradiography.

DISCUSSION

Previous studies in which the immunoblotting technique was used have localized the Zw system on platelet GP IIIa (2,4) and the platelet-specific antigens Bak^a and Lek^a on platelet GP IIb (4,10).

In this study, anti-Bak^a serum reacted with a 130-kD band under non-reducing conditions and with a 120-kD band under reducing conditions, similarly as has been described for anti-Lek^a serum (10). However, under both conditions, the anti-Lek^a serum reacted much more strongly in immunoblot than the anti-Bak^a serum, confirming results in the platelet immunofluorescence test (see also next page). Immunoblotting can be helpful for the determination of the actual allospecificity in cases like that of serum Fa that reacted serologically strong with both Zw(a+) and Zw(a-) donor platelets.

Immunoblot has not been as successful in the determination of platelet autoantigens: the reactivity with GP IIIa and a 220-250-kD protein have been reported in a limited number of sera (5,7,9). In contrast to sera containing alloantibodies, most sera containing autoantibodies and all eluates prepared from ITP platelets did not react in our immunoblot study. Only two sera (Es and Ro) showed specific reactions.

ITP serum Es reacted in immunoblot with a 130-kD band and a 240-kD band, irrespective of the Bak^a type . It reacted only with the 240-kD band of a solubilizate prepared from Glanzmann type-I platelets. To our knowledge, this is the first ITP serum in which specific reactivity with GP IIb could be demonstrated. Unfortunately, the platelet eluate of patient Es could not be tested. The serum had a strong specific reactivity in immunoblot, whereas in the immunofluorescence test the reactivity was only weak. This discrepancy might be caused by a better exposition of the antigenic site due to the immunoblot procedure.

The reactivity of serum Es and all anti-Bak^a sera diminished or disappeared when the platelet solubilizate was prepared in the presence of 5 mM Na_2EDTA. This phenomenon might be explained by the findings of Fujimura & Phillips (17) who have demonstrated that GP IIb is more readily hydrolysed in the absence of Ca^{2+} (by addition of EDTA or EGTA), probably because more GP IIb-IIIa complexes are dissociated in the presence of EDTA or EGTA. An amount as small as 10^{-3} M Ca^{2+} appeared to be enough to make GP IIb resistant for hydrolysis. Applied to our results, this could mean that more antigenic sites for Bak^a on GP IIb might have been accessible for digestion by proteases after solubilization of platelets in the presence of EDTA. This might have led to a loss of reactivity of anti-Bak^a serum for Bak(a+) platelet solubilizate. Another explanation for the loss of reactivity of anti-Bak^a serum with EDTA solubilizate might be the alteration of the antigenic determinant of Bak^a during dissociation of the GP IIb-IIIa due to EDTA.

The anti-Lek^a serum, diluted 1:20, still reacted with the 130-kD band of EDTA solubilizate. This might be explained by the many times greater reactivity of this serum compared to anti-Bak^a sera (as observed by PIFT) or by recognition of a different epitope on GP IIb. The fact that anti-Bak^a and anti-Lek^a recognize the same donor populations in platelet serology pleas against the last hypothesis (18).

ITP serum Ro, as well as the three sera containing antibodies against so-called cryptantigens (one serum containing EDTA-dependent antibodies and two sera containing PFA-dependent antibodies), reacted with a 240-kD band. Therefore, the antigen located on the 240-kD band might be a so-called cryptantigen. This band was also consistently recognized by all anti-Bak^a sera and serum Es. The reactivity with a similar band has been reported by others (5,7) and might be due to aspecific protein-protein interactions. However, the fact that, in case of ITP serum Ro, reactivity remained at a dilution of 1:100 pleas against aspecificity.

As to the nature of the band, it might represent actin-binding protein (240 kD) or the so-called p235, because platelet activation by cold as well as activity of calcium-dependent protease weakens expression of the band as shown by staining with Coomassie Brilliant Blue (19,20). Close linkage of cytoskeleton proteins with strongly antigenic surface glycoproteins has been described for actin-binding protein to GP Ib (21) and for actin to GP IIb-IIIa (22,23). This linkage might promote the development

of antibodies directed against so-called cryptantigens, which can be frequently encountered in the platelet immunofluorescence test. The fact that reactivity with the 240-kD band is frequently encountered in sera with allo- or autoantibodies directed against the strongly antigenic GP IIb might fit in this hypothesis as well.

In conclusion, most autoantisera and all eluates prepared from ITP platelets did not react with specific bands in immunoblot in contrast to alloantisera and eluates prepared from Zw(a+) donor platelets sensitized with anti-Zw^a antibodies. However, van Leeuwen et al. (24) have shown in the platelet immunofluorescence test, that most eluates prepared from ITP platelets react with normal donor platelets, but not with platelets of patients with Glanzmann type I, suggesting a specific reactivity of most autoantibodies for the GP IIb-IIIa complex.

The lack of reactivity of most autoantisera and eluates prepared from ITP platelets might be caused by the loss of conformational antigenic determinants due to dissociation of the GP IIb-IIIa complex, as Rosa et al. have suggested (25). This suggestion was further strengthened by our finding that several eluates, not reacting in immunoblot, could precipitate GP IIb-IIIa complex in a radioimmuno precipitation technique.

Further investigations are being carried out in our laboratory to elucidate the roles of protein conformation and of EDTA in antigenic expression as well as the importance of the 240-kD band as target antigen.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Nanneke J. Risseeuw-Bogaert for her technical assistance, Mrs Carla M. van der Plas-van Dalen for the supply of sera and eluates and Mrs Jetty Gerritsen for her secretarial assistance.

REFERENCES

1. Kunicki TJ, Aster RH (1979) Isolation and immunologic characterization of the human platelet allo-antigen Pl^{A1}. Mol Immunol 15: 353-360.
2. McMillan RC, Mason D, Tani P, Schmidt GM (1982) Evaluation of platelet surface antigens: localization of the Pl^{A1} allo-antigen. Brit J Haematol 51: 297-304.
3. Mulder A, Leeuwen EF van, Veenboer GJM, Tetteroo PAT, Borne AEG Kr von dem (1984) Immunochemical characterization of platelet-specific alloantigens. Scand J Haematol 33: 267-274.
4. Schoot CE van der, Wester M, Borne AEG Kr von dem, Huisman JG (1986) Characterization of platelet-specific alloantigens by immunoblotting: localization of Zw and Bak antigens. Brit J Haematol, in press.
5. Beardsly DS, Spiegel JE, Jacobs MM, Handin RI, Lux IV SE (1984) Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. J Clin Invest 74: 1701-1707.
6. Woods VL, Oh EH, Mason D, McMillan R (1984) Autoantibodies against the platelet glycoprotein IIb-IIIa complex in patients with chronic ITP. Blood 63: 368-375.
7. Mason D, McMillan R (1984) Platelet antigens in chronic idiopathic thrombocytopenic purpura. Brit J Haematol 56: 529-534.
8. Woods VL, Kurata Y, Montgomery RR, Tani P, Mason D, Oh EH, McMillan R (1984) Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood 64: 156-160.
9. Devine DV, Rosse WF (1984) Identification of platelet proteins that bind alloantibodies and autoantibodies. Blood 64: 1240-1245.
10. Kieffer N, Boizard B, Didry D, Wautier J-L, Nurden AT (1984) Immunochemical characterization of the platelet-specific alloantigen Lek^a: a comparative study with the Pl^{A1} alloantigen. Blood 64: 1212-1219.
11. Pegels JG, Borne AEG Kr von dem, Bruynes ECE, Engelfriet CP (1982) Pseudothrombocytopenia: an immunological study on platelet antibodies dependent on ethylene diamine tetra-acetate. Blood 59: 157-161.
12. Borne AEG Kr von dem, Lelie J van der, Vos JJE, Plas-van Dalen CM van der, Risseeuw-Bogaert NJ, Ticheler MDA, Pegels JG (1986) Antibodies against cryptantigens of platelets. Characterization and significance for the serologist. In: Platelet Serology, Research Progress and Clinical Implications (Décary F, Rock GA, eds), Karger, Basel, pp 33-46.
13. Helmerhorst FM, Leeuwen EF van, Pegels JG, Riesz E von, Engelfriet CP (1980) Autoimmune thrombocytopenia: detection of platelet antibodies with the suspension immunofluorescence test. Brit J Haematol 45: 319-327.
14. Boizard B, Wautier J-L (1984) Lek^a, a new platelet antigen absent in Glanzmann's thrombasthenia. Vox Sang 46: 47-54.
15. Fraker PJ, Speck JC (1980) Protein and cell membrane iodinations with sparingly soluble chloramide-1,2,4,6-tetrachloro-3α,6α-diphenyl glycouril. Biochem Biophys Res Commun 4: 849-857.
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
17. Fujimura K, Phillips DR (1982) Calcium cation regulation of glycoprotein IIb-IIIa complex formation in platelet plasma membranes. J Biol Chem 258: 10247-10252.
18. Borne AEG Kr von dem, Plas-van Dalen CM van der (1986) Bak^a and Lek^a are identical antigens. Brit J Haematol 62: 404-405.
19. Fox JEB, Goll DE, Reynolds CC, Phillips DR (1985) Identification of two proteins (actin-binding protein and p235) that are hydrolyzed by endogenous Ca^{2+}-dependent protease during platelet aggregation. J Biol

Chem 260: 1060-1066.

20. Nachmias VT, Kavaler J, Jacubowith S (1985) Reversible association of myosin with the platelet cytoskeleton. Nature 313: 70-72.

21. Okita JR, Pidard D, Newman PJ, Montgomery RR, Kunicki TJ (1985) On the association of glycoprotein Ib and actin-binding protein in human platelets. J Cell Biol 100: 317-321.

22. Phillips DR, Jennings LK, Edwards HH (1980) Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol 86: 77-86.

23. Fox JEB, Phillips DR (1983) Polymerization and organization of actin filaments within platelets. Sem Hematol 20: 243-260.

24. Leeuwen EF van, Ven JTM van der, Engelfriet CP, Borne AEG Kr von dem (1982) Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 59: 23-26.

25. Rosa J-P, Kieffer N, Didry D, Pidard D, Kunicki TJ, Nurden AT (1984) The human platelet membrane glycoprotein complex GP IIb-IIIa expresses antigenic sites not exposed on the dissociated glycoproteins. Blood 64: 1246-1253.

Vox Sang. 49: 92–100 (1985)

0042–9007/85/0492–0092 $2.75/0

Intravenous Gammaglobulin Therapy in Idiopathic Thrombocytopenic Purpura

Results with the Netherlands Red Cross Immunoglobulin Preparation

J. J. E. Vos, W. G. van Aken, C. P. Engelfriet, A. E. G. Kr. von dem Borne

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, incorporating the Laboratory of Experimental and Clinical Immunology of the University of Amsterdam, The Netherlands

Abstract. The effect of high-dose intravenous gammaglobulin (IVG) therapy with a CLB preparation was studied in 42 patients: 8 patients had acute and 26 patients had chronic idiopathic thrombocytopenic purpura (ITP); 5 patients had thrombocytopenia accompanied by various diseases such as systemic lupus erythematosus, auto-immune haemolytic anaemia and neutropenia; 3 patients had hypoplastic anaemia and 1 patient had neutropenia and rheumatoid arthritis. After treatment, a rise in platelet count occurred in about 75% of the patients with ITP, although there was no sustained response in any of the patients. There was no correlation between the strength of platelet antibodies as detected by the direct immunofluorescence test before infusion and the pattern of response to the infusion. In most cases of ITP, no immune complexes, as measured by Clq-binding assay, were observed. Furthermore, we found no relationship between the amount of Clq-binding activity of patients' sera and the reaction pattern after infusion of IVG. Splenectomy of the patient had no influence on the outcome of IVG therapy.

Introduction

Infusion of high doses of intravenous gammaglobulin (IVG) can be effective in the treatment of idiopathic thrombocytopenic purpura (ITP) [1–6]. The mechanism of this effect remains obscure, and it is not yet clear whether every preparation of IVG is effective. An immunoglobulin preparation for intravenous use was recently developed in our laboratory. We tested it for its effect in ITP. Moreover, we tried to relate various immunological and clinical parameters (i.e. platelet-bound Ig, Clq-binding assay, splenectomy) to its effectiveness.

Materials and Methods

Immunoglobulin

The immunoglobulin CLB (prepared in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) is produced from donor plasma by ethanol cryoprecipitation (Cohn fraction II). The resulting product is then treated at pH 4 with traces of pepsin to make it suitable for intravenous use. Its characteristics are similar to those of the immunoglobulin described by *Skavaril* [7] and used by *Imbach* et al. [1,

Table I. Patients with Secondary ITP

Patient	Sex	Age years	Diagnosis	Effect of treatment with IVG
A	F	28	AIHA+ITP	no effect (twice)
B	M	31	AIHA+ITP	no effect
C	M	34	HA	no effect
D	F	65	HA	no effect
E	F	45	HA	no effect
F	F	12	ITP+neutropenia	rise in platelet count; no change in leucocytes
G	M	64	RA+neutropenia	no effect
H	F	31	ITP+neutropenia +SLE	rise in platelet count; no change in leucocytes

RA = Rheumatoid arthritis; SLE = systemic lupus erythematosus; HA = hypoplastic anaemia.

2]. It contains 99% IgG, 1% IgA and traces of IgM (protein content 60 g/l). IgG subclasses are distributed as follows: IgG1 57.5%, IgG2 28%, IgG3 9%, IgG4 5.5%. 95% of the IgG is monomeric, 7% dimeric and 3% polymeric. All patients received the high-dose IVG as described by *Imbach* et al. [1, 2], i.e. daily intravenous infusions of immunoglobulin CLB, 0.4 g/kg during 5 days.

Platelet Counts

Platelet counts were determined by automatic cell counting. Platelet numbers below 50×10^9/l were determined by microscopical counting. They were followed (if possible) daily for at least 10 days after the start of treatment.

Platelet auto-antibodies were determined (if possible) before and after IVG treatment by the fluorescent antiglobulin technique on paraformaldehyde-fixed platelets (PSIFT) as described by *von dem Borne* et al. [8]. The C1q-binding assay was performed as described by *Zubler* et al. [9]. Serum IgG/IgM/IgA levels were measured before treatment. Serum IgG was also measured after each infusion.

Patients

We studied 3 groups of patients. Group 1 consisted of patients, adults as well as children, with the diagnosis chronic ITP who continued to have bleeding problems despite conventional treatment (i.e. corticosteroids, immunosuppressive drugs and/or splenectomy). The diagnosis ITP was assessed as an idiopathic bleeding disorder with a low platelet count ($<50\times10^9$/l), no splenomegaly, normal or increased numbers of megakaryocytes in a bone marrow smear in the absence of disorders responsible for secondary thrombocytopenia. This group consisted of 15 adults (5 males, 10 females) with a mean age of 40 years (range 17–75 years) with ITP during an average time of 5.5 years (7 months–18 years) and 11 children (5 males, 6 females) with a mean age of 10.5 years (2–17 years) with ITP during an average time of 2.5 years (5 months–9 years).

The adult patients had been treated daily with 60 mg prednisone for at least 3 weeks. At the time of high-dose IVG therapy 2 patients were still treated with 15 mg prednisone daily. In addition, some patients had received vincristine (n = 6), azathioprine (n = 6) and vinblastine (n = 1). 9 patients had been splenectomized and 2 were splenectomized after treatment with IVG.

The children had been treated for at least 2 weeks with prednisone, 2 mg/kg daily, without effect. At the time of the trial no children were being treated with prednisone. None had been splenectomized and no further therapy had been applied.

Group 2 consisted of 8 untreated children with an acute-onset ITP (6 males, 2 females) of whom 2 had a recurrent type of acute ITP (1 male, 1 female).

Group 3 consisted of 8 patients with a variety of diseases, mostly associated with thrombocytopenia. Among them were 2 patients with Evans syndrome, i.e. auto-immune haemolytic anaemia (AIHA) + ITP, and 2 patients with ITP in combination with a neutropenia. 3 patients had hypoplastic anaemia and 1 patient had neutropenia associated with rheumatoid arthritis (table I).

Patients' data were obtained from a questionnaire filled in by the participating clinicians.

Results

Platelet counts rose above 50×10^9/l in 11 of the 15 adult patients of group 1 (chronic ITP). In 7 of these patients platelet counts increased to more than 100×10^9/l (fig. 1a, b). The duration of response (as far as we were able to assess) varied from 4 to 42 days after the last day of infusion. No difference in response to IVG therapy was seen between the splenectomized and the non-splenectomized patients: platelet counts rose to above 50×10^9/l in 7 of the 9 splenectomized patients. In 3 of these patients, platelet counts increased to more than 100×10^9/l. In 5 of the 6 non-splenectomized patients, platelet counts rose to above 50×10^9/l, while in 3 of them they increased to more than 100×10^9/l ($p>0.05$, Fisher exact test).

In the children with chronic ITP the platelet count of 9 out of the 11 patients rose to above 50×10^9/l, and the platelet count of 8 of these 9 patients rose even above 100×10^9/l (fig. 1d). Because none of these patients was splenectomized, we could not determine the relationship between response in splenectomized versus non-splenectomized patients in this group. In 2 of these 11 patients (1 13-year-old girl and 1 11-year-old boy), haptoglobin levels and direct and indirect antiglobulin tests on erythrocytes and serum, respectively, were monitored during IVG therapy. These tests were and remained normal, although both patients reacted with a transient rise of platelet counts to above 100×10^9/l.

In group 2 (acute-onset ITP) 7 out of 8 patients showed a rise in platelet count, in 6 of them to numbers exceeding 100×10^9/l (fig. 1c). In 3 patients the increase in number of platelets was only temporary. In 1 of these patients, and in the non-reacting patient, spontaneous remission occurred later. The 2 patients with recurrent acute-onset ITP both reacted with an increase in number of platelets to above 100×10^9/l after gammaglobulin treatment which lasted for at least 41 days. In a previous episode of thrombocytopenia the platelet count in these patients recovered spontaneously.

In group 3 we observed no increase in platelet counts or in haemoglobulin levels in both patients with ITP associated with autoimmune haemolytic anaemia. In 1 patient the thrombocytopenia afterwards reacted to a combination therapy of prednisone, vincristine and azathioprine. Furthermore, in hypoplastic anaemia none of the 3 patients showed a rise in any of their blood components. The number of leucocytes did not rise in any of the 3 patients with a neutropenia. However, in 2 patients with neutropenia the associated thrombocytopenia disappeared and a rise in platelet count to above 100×10^9/l was seen.

Platelet Auto-Antibodies

We found no relationship between pretreatment results of PSIFT and efficacy of gammaglobulin treatment, in patients with chronic ITP or with acute-onset ITP (table II, fig. 2). Even 1 out of 2 patients in whom no platelet auto-sensitization was observed in the PSIFT showed an increase in the number of platelets to above 50×10^9/l. Also, 1 patient with only IgM auto-antibodies reacted, whereas another did not. In 7 of the 15 adult patients with chronic ITP we

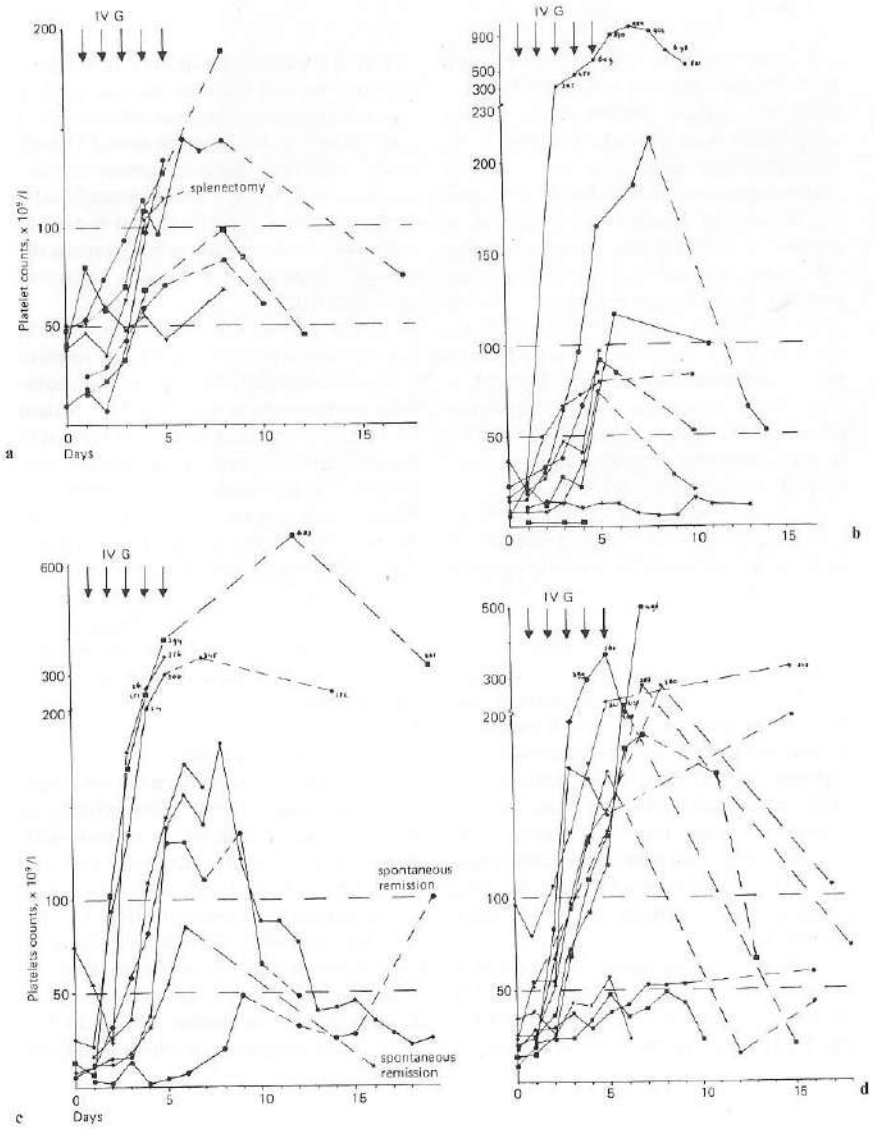

Fig. 1. Platelet count curves in non-splenectomized adults with chronic ITP (**a**), splenectomized adults with chronic ITP (**b**), children with (recurrent) acute ITP (**c**) and children with chronic ITP (**d**).

Table II. Relation between classes of auto-antibodies as determined in PSIFT and effectiveness of IVG (in patients with acute and chronic ITP; n = 28)

Ig class	Maximum platelet response		
	0–50 $\times 10^9$/l	50–100 $\times 10^9$/l	100 $\times 10^9$/l
IgG		3	4
IgM	1		1
IgG+M	2	1	8
IgG+A+M	1	1	2
IgA+M			1
No observable auto-antibody	1		1
No specification	1		

performed the PSIFT before and after treatment with gammaglobulin. The serological results were not significantly affected by the treatment. In 6 of the 8 children with chronic ITP, pre- and posttreatment tests were performed with similar results.

Clq-Binding Assay

In 15 patients with chronic ITP the Clq-binding assay was performed, in 8 before as well as after treatment. In 5 patients a small elevation of circulating immune complexes was observed before therapy (fig. 3). In 2 patients this normalised after treatment. In 2 patients the percentage of Clq-binding activity was not changed. 1 patient was not tested afterwards. Furthermore, 1 patient's negative Clq-binding assay became positive after treatment.

Serum Immunoglobulins

None of the patients showed an IgA deficiency. IgG levels rose from an average of 12.6 (8–19.4) to 31.9 g/l (17.6–55.2) (n = 16) during treatment.

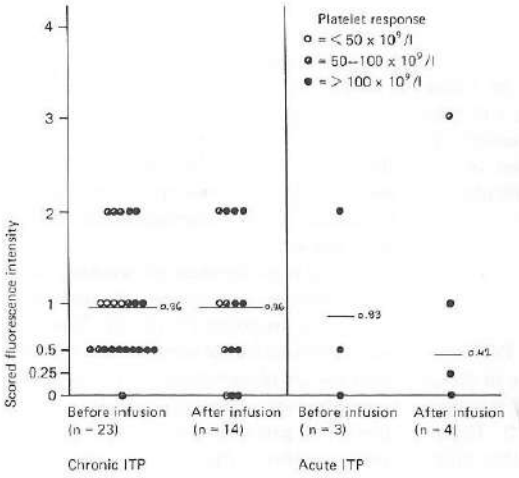

Fig. 2. Results of PSIFT in patients with ITP before and after IVG therapy. The intensity of fluorescence was recorded as ++++, +++, ++, +, (+), ±, and transformed into the following values: 4, 3, 2, 1, 0.5, 0.25.

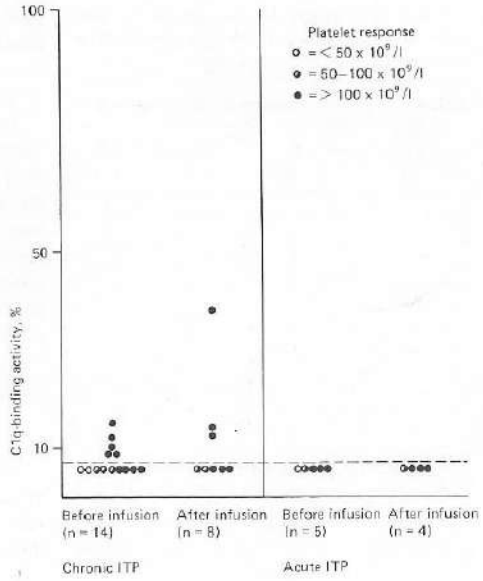

Fig. 3. Results of performance of the Clq-binding assay in patients with ITP before and after IVG therapy.

Side Effects

3 patients reported headache, in 1 case associated with nausea, vomiting and subfebrile temperature. In this last patient the complaints led to discontinuation of the treatment after 2 days, however, the platelet count rose to above 100×10^9/l.

Discussion

The above results show that IVG prepared in our institute was effective in about 75% of the patients with chronic ITP (rise of platelet counts to above 50×10^9/l). Therefore, it is as effective as various other preparations studied [1–5]. The efficacy of therapy was shown not only by a rise in the number of platelets, but also by a normalisation of the bleeding time as far as determined (data not shown). Good platelet function was confirmed by uncomplicated surgery of 4 patients after gammaglobulin therapy (data not shown).

Several hypotheses on the working mechanisms of high-dose gammaglobulin therapy have been proposed [3, 10, 11]. The most plausible explanation seems to be a transient blockade of phagocytosis in the mononuclear phagocytic system, especially in the spleen, by gammaglobulin. *Fehr* et al. [3] confirmed this by finding a concomitant depression of the clearance of radio-labelled erythrocytes sensitized with IgG antibodies during successful gammaglobulin therapy in chronic ITP.

Other working mechanisms may be that immune complexes bound to the platelet Fc-receptor are competitively eliminated by immunoglobulin [12–14] or that immunoglobulin, which because of its polyspecificity contains anti-idiotype antibodies, causes auto-antibody production to be stopped [4, 16].

We tried to measure these phenomena by testing changes in the strength of the direct immunofluorescence test on platelets and circulating immune complexes after successful treatment [15], but we could not confirm either of these mechanisms because platelet auto-antibodies remained demonstrable to the same extent after treatment and in only a few patients was a slightly elevated Clq-binding assay measured (fig. 2, 3).

We could not determine a relationship between pretreatment results of the platelet auto-antibody test and a positive effect of gammaglobulin (fig. 2, table II). Even a patient with no demonstrable auto-antibodies reacted. Auto-antibodies may have been present, but in too low a concentration to be detectable.

Salama et al. [11] have proposed a new hypothesis for the working mechanism of IVG, namely it induces a mild, clinically non-apparent haemolysis by which the mononuclear phagocytic system would preferentially be blocked. Therefore, in 2 of our patients, we monitored haptoglobin levels, as well as direct and indirect antiglobulin tests on erythrocytes and serum, respectively, but these tests were and remained normal, although both patients reacted with a transient rise in platelet counts above 100×10^9/l. This, as well as the frequent occurrence of ITP together with AIHA in Evans' syndrome and the absence of reaction on IVG in patients with Evans' syndrome, argues strongly against this hypothesis. Other authors have also reported evidence against this hypothesis [17–19].

Searching for a parameter of successful treatment, *Schmidt* et al. [5, 6] have suggested a relationship between splenectomy and successful treatment. From our results, it seems that patients with or without splenectomy reacted equally well. Hence, we could not confirm *Schmidt's* finding that IVG is more effective in splenectomized patients.

We have shown, like others [1, 2], that IVG can be effective in acute-onset ITP, but contrary to the results of others, not always. When studying the effect of gammaglobulin in acute-onset ITP, one has to bear in mind the natural history of this disease: 80% show a spontaneous recovery. This fact was illustrated by 1 spontaneously recovering patient, although he had not reacted to IVG therapy, and by 1 patient who, after a transient rise in platelet count, had spontaneously recovered. Thus, the only way of showing a real effect of gammaglobulin therapy in acute-onset ITP will be a double-blind (cross-over) study.

IVG can also be effective in secondary ITP. A temporary rise in platelet count was seen in patients with ITP associated with systemic lupus erythematosus and in patients with ITP associated with neutropenia. However, no rise in platelet count was seen in the 2 patients with Evans' syndrome (AIHA + ITP). Successful treatment with IVG in neutropenia has been reported [20, 21]. However, we found no rise in neutrophil

counts in 3 patients with neutropenia, although the concomitant thrombocytopenia in 2 of these 3 patients clearly reacted.

In conclusion, we found that CLB-IVG was as effective in patients with ITP as were other preparations. However, the therapeutic use of gammaglobulin in general should be restricted to emergency cases such as intercranial or large gastro-intestinal bleedings, because of its mostly temporary effect and high costs of treatment on the one hand, and because of the low bleeding tendency in patients with low platelet counts on the other hand.

Acknowledgements

We are indebted to the participating physicians for their willing cooperation. We thank Mrs. *Carla van der Plas-van Dalen* and co-workers for performing the PSIFT, Mrs. *Hanneke de Wolff, M. P. J. Piët* and *J. P. P. Moors* for the accurate filling in of the patients' data, and Mrs. *Wanda Winkel* and Mrs. *Jetty Gerritsen* for the careful preparation of the typescript.

References

1 Imbach, P.; Barandun, S.; d'Apuzzo, V.; Baumgartner, C.; Hirt, A.; Morell, A.; Rossi, E.; Schöni, M.; Vest, M.; Wagner, H.P.: High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura in childhood. Lancet *i:* 1228–1231 (1981).

2 Imbach, P.; Barandun, S.; Baumgartner, C.; Hirt, A.; Hofer, F.; Wagner, H.P.: High-dose intravenous gammaglobulin therapy of refractory, in particular idiopathic thrombocytopenia in childhood. Helv. paediat. Acta *46:* 81–86 (1981).

3 Fehr, J.; Hofmann, V.; Kappeler, U.: Transient reversal of thrombocytopenia in idiopathic thrombocytopenic purpura by high-dose intravenous gammaglobulin. New Engl. J. Med. *306:* 1254–1258 (1982).

4 Newland, A.C.; Treleaven, J.J.; Minchinton, R.M.; Waters, A.H.: High-dose intravenous IgG in adults with auto-immune thrombocytopenia. Lancet *i:* 84–87 (1983).

5 Schmidt, R.E.; Budde, U.; Schäfer, G.; Stroehmann, I.: High-dose intravenous gammaglobulin for idiopathic thrombocytopenic purpura. Lancet *ii:* 475–476 (1981).

6 Schmidt, R.E.; Budde, U.: Erfolgreiche Behandlung von Immunothrombopenien mit hochdosierten intravenösen Immunoglobulinen; in Schmidt, Strochmann, Immunoglobulintherapie. Beitr. Infusionstherapie klin. Ernähr., vol. 11, pp. 73–78 (Karger, Basel 1983).

7 Skavaril, F.: Qualitative and quantitative aspects of IgG subclasses in i.v. immunoglobulin preparations; in Nydegger, Immunohemotherapy: a guide to immunoglobulin prophylaxis and therapy, pp. 113–122 (Academic Press, London 1981).

8 Borne, A.E.G. Kr. von dem; Verheugt, F.W.A.; Oosterhof, F.; von Riesz, L.E.; Brutel de la Rivière, A.; Engelfriet, C.P.: A simple immunofluorescence test for the detection of platelet antibodies. Br. J. Haemat. *39:* 195–207 (1978).

9 Zubler, R.H.; Lange, G.; Lambert, P.H.; Miescher, P.A.: Detection of immune complexes in unheated sera by a modified ^{125}I-Clq binding test. J. Immun. *116:* 232–235 (1976).

10 Imbach, P.; Jungi, T.W.: Possible mechanism of intravenous immunoglobulin treatment in childhood idiopathic thrombocytopenic purpura (ITP). Blut *46:* 117–124 (1983).

11 Salama, A.; Mueller-Eckhardt, C.; Kiefel, V.: Effect of intravenous immunoglobulin in immune thrombocytopenia. Lancet *ii:* 193–195 (1983).

12 Lurhuma, A.Z.; Riccomi, H.; Mason, P.L.: The occurrence of circulating immune complexes and viral antigen in idiopathic thrombocytopenia. Clin. exp. Immunol. *28:* 49–55 (1977).

13 Trent, R.J.; Clancy, R.L.; Danis, V.; Barten, A.: Immune complexes in thrombocytopenic patients: cause or effect? Br. J. Haemat. *44:* 645–654 (1980).

14 Ercilla, M.J.; Borche, L.; Vives, J.; Castillo, R.; Gelabert, A.; Rozmann, C.: Circulating immune complexes in immune thrombocytopenic purpura (ITP). Br. J. Haemat. *52:* 679–680 (1982).

15 Schmidt, B.; Forster, J.: Increased platelet associated IgG in child on high-dose gammaglobulin for idiopathic thrombocytopenic purpura. Lancet *ii:* 39–40 (1982).

16 Tsubakio, T.; Kurata, Y.; Katagiri, S.; Kanakura, Y.; Tamaki, T.; Kuyama, J.; Kanagama, Y.; Yonezawa, T.; Tarai, S.: Alteration of T-cell subsets and immunoglobulin synthesis in vitro during high-dose gammaglobulin therapy in patients with idiopathic thrombocytopenic purpura. Clin. exp. Immunol. *53:* 697–702 (1983).

17 Imbach, P.; Jungi, T.W.; Barandun, S.: Intravenous immunoglobulin in immune thrombocytopenia. Lancet *ii:* 577 (1983).

18 Marmont, A.M.: Intravenous immunoglobulin in immune thrombocytopenia. Lancet *ii:* 577–578 (1983).

19 Budde, U.; Schäfer, S.; Niese, D.; Schmidt, R.E.: Intravenous immunoglobulin in immune thrombocytopenia. Lancet *ii:* 578 (1983).

20 Pollack, S.; Cunningham-Rundles, C.; Smithwick, E.M.; Barandun, S.; Good, R.A.: High-dose gammaglobulin for auto-immune neutropenia. New Engl. J. Med. *307:* 253 (1982).

21 Bussel, J.; Lalezari, P.; Hiltgardner, M.; Partin, J.; Fikrig, S.; O'Malley, J.; Barandun, S.: Reversal of neutropenia with intravenous gammaglobulin in auto-immune neutropenia of infancy. Blood *62:* 398–400 (1983).

Received: July 11, 1984
Accepted: November 28, 1984

Dr. J.J.E. Vos,
Central Laboratory of the Netherlands
Red Cross Blood Transfusion Service,
PO Box 9190,
1006 AD Amsterdam (The Netherlands)

SUMMARY

This thesis deals with some aspects of diagnosis and treatment of idiopathic thrombocytopenia (ITP).

Chapter I contains an introduction to the subject by means of a short review of general insights in diagnosis and therapy of ITP. Because ITP is generally considered to be an autoimmune disease, many assays measuring platelet-associated immunoglobulins and most of them measuring platelet-associated IgG (PA-IgG), have been developed for diagnostic purposes. PA-IgG values for normal healthy donor platelets as well as for patients' platelets vary considerably between the various assays reported.

In Chapter II, a recently developed radioimmunoassay is described, by which several variables that may influence accurate PA-IgG measurement were studied. Mean PA-IgG in normal donor platelets appeared to decrease upon storage in buffer containing EDTA, or after fixation of platelets with paraformaldehyde (PFA). Simultaneously with this decrease, an increase in modal platelet volume was observed. Therefore, the decrease in PA-IgG was postulated to be caused by release of plasma IgG, entrapped in the surface-connected canalicular system, due to swelling of the platelets during storage in EDTA buffer or after PFA fixation. Good quantification of platelet-bound alloantibodies appeared to be possible using the radioimmunoassay. Aspecifically increased PA-IgG was observed when fragments of platelets or other cell types were present in test samples. It was concluded that cellular fragments can interfere with proper PA-IgG measurement in PA-IgG assays of this type.

In Chapter III, the sensitivity and specificity of the platelet immunofluorescence test (PIFT) for the diagnosis of ITP was studied in a consecutive series of 255 patients. The sensitivity for the diagnosis ITP was high, whereas the specificity was low. This low specificity was not only due to the occurrence of ITP with other diseases, but also because of the frequent occurrence of EDTA- and PFA-dependent antibodies.

From the results described in Chapter IV, the radioimmunoassay for PA-IgG appeared to have about similar sensitivity and specificity for the diagnosis ITP compared to the PIFT. A significant correlation was observed between the mean amount of PA-IgG measured and the height of PIFT scores with anti-IgG. A more frequent incidence of aberrant size distribution

plots was seen in patients with increased PA-IgG, indicating a possible influence of platelet fragments on the measurement of PA-IgG. In patients with increased PA-IgG and a negative PIFT with anti-IgG, IgM antibodies were detected more frequently in the PIFT. This suggests an influence on PA-IgG determinations of either IgM antibody-induced platelet agglutination *in vitro* or of platelet fragments present in the patient's blood because of IgM antibody-mediated platelet destruction *in vivo*.

In Chapter V, the existence of platelet fragments in platelet-rich plasma of thrombocytopenic patients could be demonstrated by a radioimmunoassay (RIA), using a sandwich of two monoclonal antibodies directed against the platelet-specific glycoprotein IIb-IIIa complex (GP IIb-IIIa). Platelets and platelet fragments were separated by the use of a discontinuous 7.5-20% (v/v) albumin gradient.

Platelet contamination in filtered red blood-cell concentrates was studied with the same technique as described in Chapter V. From the results described in Chapter VI, it appeared that platelet fragmentation may occur in the production of a leukocyte-poor red blood-cell concentrate. Therefore, even when no intact platelets pass through the filter, platelet antigenic material may find its way into the red blood-cell concentrate. This finding might have important implications for the preparation of filtered red blood cells. Unwanted immunization might occur after transfusion, especially when not only platelets but also leukocytes would be fragmented during the preparation of leukocyte-poor red blood-cell concentrates.

In Chapter VII, preliminary results of a study on specific antigens for platelet autoantibodies are presented. For this purpose, the immunoblotting technique was used, by which specific binding of platelet antibodies to platelet membrane protein bands can be visualized. With this technique, good visualization of target proteins for platelet alloantibodies can be obtained. Therefore, it is a suitable technique for the determination of the actual specificity in sera with troublesome serological typing.

In the study, only 2 out of 24 ITP sera tested reacted in immunoblot. Moreover, none of 12 eluates prepared from ITP platelets (all reacting strongly with donor platelets in PIFT) reacted in immunoblot. Because 5 of these eluates were able to precipitate GP IIb-IIIa in a radioimmunoprecipitation technique, these results may indicate a loss of conformational determinants for autoantibodies during the immunoblotting procedure. Furthermore, the reactivity in immunoblot appeared to be strongly influenced by circumstances before or during solubilization of donor platelets (presence of EDTA and N-ethylmaleamide; platelet activation by cold).

Finally, in Chapter VIII, the results of treatment of ITP with The Netherlands Red Cross immunoglobulin preparation are described. A mostly transient rise in platelet count was observed in about 75% of the patients with ITP. There was no correlation between the strength of PIFT or the amount of C1q-binding activity in the patient's serum before infusion and the response to intravenous gammaglobulin therapy. Splenectomy in the patient's history did not increase the predictability of a beneficiary effect on the outcome of intravenous gammaglobulin treatment.

SAMENVATTING

In dit proefschrift worden enige aspecten van de diagnostiek en behandeling van idiopathische thrombocytopenische purpura (ITP) onder de loep genomen.

Hoofdstuk I is een algemene inleiding op het onderwerp, waarin een kort overzicht wordt gepresenteerd van de huidige inzichten ten aanzien van diagnose en therapie van ITP. ITP wordt in het algemeen tot de autoimmuunziekten gerekend. Daarom zijn er voor de diagnostiek van ITP een groot aantal testen ontwikkeld, waarmee de hoeveelheid plaatjes (=thrombocyten)-geassocieerde immunoglobulinen wordt gemeten. De meeste van deze testen registreren slechts plaatjes-geassocieerd IgG (PA-IgG). Deze testen verschillen onderling aanzienlijk zowel in normaalwaarden van donorthrombocyten als in waarden gemeten bij thrombocytopenische patiënten.

In Hoofdstuk II worden de resultaten van een onderzoek beschreven naar enige variabelen, welke van invloed zijn op de bepaling van PA-IgG. De gemiddelde PA-IgG waarde van normale donorthrombocyten bleek, getest in een recent ontwikkelde radioimmunoassay, te dalen wanneer de thrombocyten eerst een tijd in EDTA buffer werden bewaard of wanneer zij voor de bepaling waren gefixeerd met paraformaldehyde (PFA). Tegelijkertijd met deze daling bleek het modale plaatjesvolume in grootte toe te nemen. De mogelijke oorzaak van deze daling van PA-IgG zou het vrijkomen van plasma IgG uit het zg. 'surface-connected canalicular system' kunnen zijn, wat tengevolge van de zwelling van de thrombocyt onder invloed van het EDTA of PFA in het thrombocytenoppervlak wordt opgenomen. Indien men PA-IgG direct na thrombocytenbereiding zonder PFA fixatie meet, dan vindt deze uitstoting van IgG in het incubatiemedium plaats met als gevolg dat een grotere hoeveelheid IgG gemeten wordt en dientengevolge een hogere PA-IgG waarde. Met deze radioimmunoassay bleek het goed mogelijk om hoeveelheden thrombocytgebonden alloantistoffen te meten. Verder bleek de aanwezigheid van fragmenten van thrombocyten of andere celtypes in de te testen monsters een aspecifiek verhoogde PA-IgG waarde te kunnen veroorzaken.

In Hoofdstuk III wordt een onderzoek beschreven naar de sensitiviteit en specificiteit van de immunofluorescentie test op thrombocyten (PIFT) voor de diagnose ITP. De sensitiviteit voor ITP bleek hoog, de specificiteit laag te zijn. Deze lage specificiteit was niet alleen het gevolg van

een frequente coïncidentie van autoimmuun thrombocytopenie met andere ziekten, maar ook van het frequent voorkomen van zg. EDTA- en/of PFA-afhankelijke antistoffen.

Sensitiviteit en specificiteit van de radioimmunoassay ter bepaling van PA-IgG bleken in dezelfde orde van grootte te liggen als die van de PIFT (Hoofdstuk IV). Er bleek een significante correlatie tussen de gemiddelde hoeveelheid PA-IgG en de hoogte van de PIFT-scores met anti-IgG te bestaan. Er werden vaker afwijkende grootte-verdelingen van de thrombocytenpopulatie gevonden bij patiënten met een verhoogde PA-IgG, wat zou kunnen wijzen op een invloed van thrombocytenfragmenten op de PA-IgG bepaling. Daarnaast werden er vaker IgM antistoffen aangetoond bij patiënten met een verhoogde PA-IgG waarde bij een negatieve PIFT met anti-IgG. Dit zou kunnen wijzen op beïnvloeding van de PA-IgG bepaling door hetzij *in-vitro* thrombocytenagglutinatie geïnduceerd door IgM antistoffen, hetzij de aanwezigheid van thrombocytenfragmenten ontstaan tengevolge van IgM antistoffen-gemedieerde thrombocytendestructie *in vivo*.

In Hoofdstuk V wordt het bestaan van fragmentaire thrombocyten in plaatjes-rijk plasma van thrombocytopenische patiënten aangetoond met behulp van een radioimmunoassay waarmee thrombocyten en thrombocytenfragmenten kunnen worden gedetecteerd middels een sandwich van twee monoclonale antistoffen, welke gericht zijn tegen het thrombocyt-specifieke glycoproteïne IIb-IIIa complex (GP IIb-IIIa). Scheiding van thrombocyten en thrombocytenfragmenten werd verkregen door gebruik te maken van een discontinue albumine gradiënt (7.5-20%, v/v).

Met behulp van de in Hoofdstuk V beschreven techniek bleek het mogelijk in erythrocytenconcentraten contaminatie met thrombocyten vast te stellen (Hoofdstuk VI). Hierbij bleek deze techniek erg gevoelig ten opzichte van metingen met een electronische deeltjesteller. Tevens bleek er tijdens de bereiding van een erythrocytenconcentraat fragmentatie van thrombocyten op te treden. Dit kan van belang zijn voor de bereiding van erythrocytenconcentraten, omdat ongewenste immunisatie kan optreden tengevolge van transfusie met een dergelijk product, met name wanneer naast fragmentatie van thrombocyten ook fragmentatie van leukocyten zou optreden gedurende de bereiding van het leukocyt-arme erythrocytenconcentraat.

In Hoofdstuk VII staan de resultaten beschreven van een onderzoek naar

thrombocyt-specifieke antigenen. Hierbij werd gebruik gemaakt van een immunoblotting techniek, waarmee het mogelijk is specifieke binding van antistoffen aan een bepaald eiwit te visualiseren. Met name blijkt dit goed mogelijk te zijn voor het bepalen van de antigeniciteit van bepaalde thrombocyten alloantistoffen. Daardoor is deze techniek uitermate geschikt om specificiteit te bepalen van alloantisera welke serologisch moeilijk typeerbaar blijken.

Van de 24 geteste autoantisera bleken er maar twee in de immunoblot te reageren. Bovendien bleek geen enkel eluaat gemaakt van ITP thrombocyten (welke alle een sterk positieve reactie in de PIFT vertoonden) te reageren in de immunoblot. Vijf van deze eluaten bleken wel in een immunoprecipitatie techniek GP IIb-IIIa te kunnen precipiteren, wat zou kunnen wijzen op een verlies van de antigene determinant tijdens de immunoblot procedure, waarbij een specifieke conformatie verloren kan zijn gegaan. De reactiviteit in de immunoblot techniek bleek in dit onderzoek erg afhankelijk van omstandigheden vóór en tijdens de bereiding van het thrombocytensolubilizaat (aanwezigheid van EDTA en N-ethylmaleamide; koude-activatie van thrombocyten).

In Hoofdstuk VIII worden de resultaten van behandeling van ITP met het CLB-immunoglobulinepreparaat beschreven. Er werd een -veelal tijdelijke- stijging van het thrombocytenaantal gezien bij ongeveer 75% van de ITP patiënten. Er bleek geen relatie te bestaan tussen de sterkte van de PIFT of een positieve C1q-binding test vóór infusie en de respons op gammaglobuline therapie. Splenectomie in het verleden bleek evenmin de voorspelbaarheid van een gunstige reactie op gammaglobuline therapie te vergroten.

STELLINGEN

1. De meeste testen ter kwantificering van plaatjes geassocieerd IgG meten te hoge waarden, omdat ook 'non-immuun' IgG opgeslagen in het 'surface connected canalicular system' gemeten wordt.
dit proefschrift

2. De aantoonbaarheid van glycoproteine IIb-IIIa complexen op monocyten berust op adhaesie van thrombocyten en/of thrombocytaire fragmenten gedurende de isolatie van monocyten en niet op synthese van het glycoproteine IIb-IIIa complex door de monocyt zelf.
Clementson et al., J.Exp.Med. 161: 972, 1985.

3. Haemophilie-patienten, die momenteel onverhitte factor VIII- of factor IX-preparaten gebruiken, zijn door hun behandelend arts niet goed voorgelicht omtrent de daarmee gepaard gaande risico's.
Rouzioux et al., Lancet i: 271, 1985.

4. Indien voor een vergrote, niet pathologisch aanvoelende halsklier geen keel-, neus- of oorheelkundige verklaring kan worden gevonden, moet worden gedacht aan een extraparotidale localisatie van een Whartin tumor.
Snyderman et al., Otolaryngol.Head Neck Surg. 94: 169, 1986.

5. Aanwezigheid van anti-cytoplasmatische antistoffen in het serum van een patient kan van doorslaggevend belang zijn voor het stellen van de diagnose Morbus Wegener.
Van der Woude et al., Lancet i: 425, 1985.

6. Bij de geringste verdenking op een afwijkende positie van de stapes-prothese na stapedectomie, dient een high resolution CT-scan van het rotsbeen gemaakt te worden.

7. De gunstige invloed van een goede functie van de buis van Eustachius op de helderheid van het gehoor bij perceptief slechthorenden moet niet onderschat worden.

8. Het feit dat een hoortoestel als een prothese wordt beschouwd en een bril hoogstens als een ongemak is exemplarisch voor de samenhang tussen de sociale acceptatie en het vóórkomen van een handicap.

9. Een acuut idiopathisch gehoorsverlies berust veelal op een viraal infect en behoeft daarom geen therapie.
Schuknecht et al., Arch.Otorhinolaryngol. 243: 1, 1986.

10. Het feit dat de wet een gehuwde vrouw slechts de bevoegdheid geeft haar eigen naam al of niet door de naam van haar echtgenoot te laten voorafgaan is, blijkens de problemen die zij bij het niet gebruik maken van deze bevoegdheid ondervindt, onvoldoende bekend.

11. De stelling van mr. C.J.J.M. Stolker, dat artsen die hun poliklinische patienten langer dan twintig tot dertig minuten laten wachten schade-plichtig zijn (Medisch Contact 40: 1247, 1985), behoeft aanvulling: Ook patienten die zonder opgave van reden hun poliklinische afspraak niet nakomen zijn schadeplichtig.

12. Plaatjes vullen gaatjes.

13. Het is altijd beter iets goed in de oren te knopen dan achteraf te doen alsof de neus bloedt. Het laatste wil namelijk nogal eens in het verkeerde keelgat schieten.

14. Stellingen van promovendi zijn vaak gezocht.

Stellingen behorende bij het proefschrift:
" Platelet-associated IgG and idiopathic thombocytopenic purpura "
door J.J.E. Vos

Amsterdam, 19 juni 1986

J.J.E. VOS

PLATELET-ASSOCIATED IgG and IDIOPATHIC THROMBOCYTOPENIC PURPURA