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Platelet function to estimate the bleeding risk in autoimmune thrombocytopenia

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Abstract

Background Knowledge of platelet function may assist in patient care in chronic autoimmune thrombocytopenia (cAITP).

Materials and methods We evaluated the association of platelet function with haemorrhage in 41 patients, median age 41 years (range 14–82 years, 24 females) with chronic autoimmune thrombocytopenia (cAITP). Samples were investigated for platelet P-selectin, and adhesion and aggregate formation under high shear conditions. Data were compared to those from 28 healthy controls (median age 39 years, range 23–70 years, 17 females) and correlated with a bleeding score of 0 (no bleeding) to 3 (overt mucosal bleedings).

Results P-selectin levels were higher in patients than in controls ($P < 0{\cdot}0004$). Compared to controls, the patients' samples responded to high shear with decreased adhesion to the polystyrene surface ($P < 0{\cdot}0001$), but formed aggregates of normal size. P-selectin expression was neither correlated with platelet counts, nor platelet adhesion, nor the bleeding score. Only the size of formed aggregates correlated with P-selectin ($P = 0{\cdot}01$). Platelet counts (odds ratio 0·5, 95% confidence interval 0·22–0·88; $P = 0{\cdot}04$) and adhesion (odds ratio 0·45, 95% confidence interval 0·17–0·87; $P = 0{\cdot}04$) were independently inversely correlated with bleeding symptoms.

Conclusion Platelet adhesion correlates with bleeding symptoms, while the size of aggregates that are formed under high shear correlates with *in vivo* platelet activation. The determination of these parameters may assist in estimating an individual bleeding risk and thus a decision for treatment.

Keywords Autoimmune thrombocytopenia, bleeding score, cone and plate analyzer, high shear condition, platelet function.
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Introduction

A comprehensive review covering current understanding of the pathomechanisms involved in chronic autoimmune thrombocytopenia (cAITP) was published a few years ago and was further updated recently [1,2]. Platelet autoantibodies play a key role in the premature platelet sequestration, and cellular mechanisms also have a significant role [3,4]. Various targets for antibodies have been specified, and most antibodies bind to the glycoprotein (GP) complex IIb/IIIa and/or GPIb/IX [5]. These GPs play a key role in platelet haemostasis and it has been shown that autoantibodies directed against these GPs may promote or hinder platelet function [6–11], even at normal platelet counts [6,8]. However, the function of patients' autologous platelets has not been investigated systematically.

As the majority of patients with cAITP have very low platelet counts most methods fail to evaluate the status of activation and function of their autologous platelets. Flow cytometric evaluation of platelet function allows the investigation of very few platelets, but without high shear conditions, and may still require manipulations to enrich for platelets. To circumvent these limitations, serum from cAITP patients was added to donor platelets to determine

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its influence on their function [12]. However, this indirect approach only, in part, substitutes for the direct evaluation of the patient's own platelets.

Recently, a cone and plate analyzer, the Impact-R, became commercially available for the estimation of platelet function. This device allows evaluation of platelet function under close to physiological conditions in a whole blood assay. Platelet adhesion and aggregation are tested in anticoagulated blood under arterial flow conditions and results are evaluated using an image analyzer [13].

Using this device we were interested in determining, in a pilot study, whether platelet adhesion and aggregate formation are related to the patients' degree of haemorrhage.

Materials and methods

Patients

The clinical characteristics of the 41 patients are shown in Table 1. The diagnosis of cAITP was based on clinical criteria [1,2,14]. The diagnosis of cAITP was also in line with normal or increased numbers of megakaryocytes and/or a transient response to steroids and/or intravenous immunoglobulins. Patients entered consecutively to the study and were seen in the outpatient department. They underwent a thorough clinical investigation with particular attention to signs of haemorrhage. The bleeding tendency was scored blinded to any results. It was based on the clinical investigation and on a standardized questionnaire. Scores were 0 (no signs of bleeding) to 5 (threatening or fatal) [15]. Patients from this cohort had a score of 0–1 (no or only minor signs of bleeding) to 2 or 3 (many petechiae or overt mucosal bleeding). Routine laboratory evaluations included, for all cAITP patients and controls, a complete blood count and differential blood count, lactate dehydrogenase, alanine-aminotransferase, albumin, creatinine, antinuclear antibodies, examination for human immunodeficiency virus, hepatitis B and C virus, activated partial thromboplastin time, prothrombin time, and protein in urine examination. Two patients had a transient treatment-induced normal platelet count (one after splenectomy, the other in response to high-dose steroids). Twenty-eight unrelated healthy individuals (17 females), median age 39 years, range 23–70 years, from the same geographic region served as controls. Informed consent was obtained from all individuals before entry into the study, which had been approved by the Ethics Committee of the Medical University of Vienna.

Table 1 Clinical characteristics of patients with chronic autoimmune thrombocytopenia

Age, median (range)	41 (14–82)
Sex, female/male	24/17
Platelet count ($\times 10^9$ L^{-1}), median (range)	42 (3–223)
Disease duration (years), median (range)	7 (1–32)
Lowest recorded platelet count ($\times 10^9$ L^{-1}), median (range)	16 (1–50)
Current treatment (*n*)	
prednisone	12
high-dose dexamethasone	1
Previous treatments (*n*)	
prednisone	34
intravenous immunoglobulin	15
high-dose dexamethasone	4
anti-D globulin	4
vincristine	2
splenectomy	4
Bleeding score at study entry (*n*)	
0	13
1	12
2	10
3	6
Detectable platelet antibodies (*n*)	14
GPIIb/IIIa	5
GPIb/IX	2
GPIIb/IIIa and GPIbIX	7
Antinuclear antibodies positive	3

Flow cytometry

Trisodium citrate anticoagulated whole blood (9 parts of whole blood, 1 part of trisodium citrate 0·108 mol L^{-1}) was used for all investigations by flow cytometry and the Impact-R. The determination of the expression of P-selectin by flow cytometry followed published recommendations [16] and has been reported previously [17]. Different to our previous protocol, we used platelet rich plasma rather than whole blood for this study. These changes were necessary as results were not reproducible if platelet counts were less than 60–80 $\times 10^9$ L^{-1}, but improved considerably using platelet rich plasma (coefficient of variation < 10% in 10 replicate investigations). All antibodies were used at optimal concentrations as determined by titration. Anti-CD42a (clone Beb1), Perdidine chlorophyll protein-conjugated was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA). Anti-CD62p (anti-P-selectin; clone CLB-Thromb6, Phycoerythrin-conjugated) was from Immunotech (Beckman Coulter, Fullerton, CA, USA). Isotype-matched controls were used for the determination of non-specific binding.

Platelet function under high shear condition

Platelet function under high shear condition was determined with the commercially available system Impact-R (DiaMed, Cressier, Switzerland), based on the Cone and Plate(let) Analyzer [18]. Samples were tested one to 2 h after the blood had been drawn under shear-stress (2050/s) using a specially developed Teflon cone. Plates were then washed with tap water, and stained with May – Grünwald solution according to the manufacturer's manual. Samples were analyzed with an inverted light microscope connected to an image analyzing system (Galai, Migdal Haemek, Israel). Platelet adhesion was recorded by examination of the percentage of total area covered with platelets, designed surface

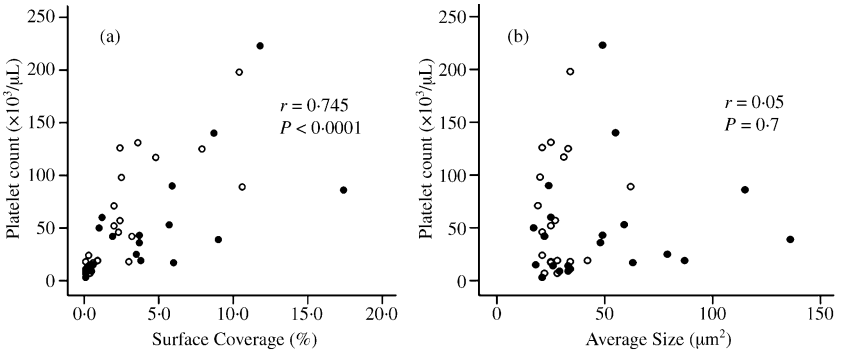

Figure 1 Platelet counts and high shear-induced platelets adhesion and size of aggregates. (a) Whole blood samples were tested under high shear conditions and results are shown as Surface Coverage for adhesion, and (b) the Average Size of the objects in 41 patients with chronic autoimmune thrombocytopenia. Full symbols for P-selectin levels > 23%, open symbols for P-selectin levels < 23%.

coverage (SC,%), and aggregates were estimated by their average size (AS, μm^2). Seven images were collected from each sample and medians of these were calculated. All samples were tested by the same person (B.E), to reduce the variation from one operator to the next. Thereby, an intra-assay coefficient of variation (CV) of 10% ($n = 15$), and a day-to-day variation of 15% were seen for the SC ($n = 10$), and both CV were 10% for the AS.

Determination of platelet antibodies

Platelet antibodies against GPIIb/IIIa and GPIb/IX were determined in all patients by monoclonal antibody-specific immobilization of platelet antigens test using the patients' autologous platelets, as described [19,20].

Statistical analyses

The Chi-squared test was used for comparison of categorical variables between groups. The Mann–Whitney-*U*-test was applied to compare metric variables between two groupsand Kruskal–Wallis test to compare more than two groups. Correlations were calculated with non-parametric Spearman test. Univariate and multivariate logistic regression analyses were used to estimate if SC and AS are associated with bleeding independent from platelet counts. Odds ratios (OR) were calculated for changes of platelet counts of 10×10^9 L^{-1}. A two-sided *P*-value < 0·05 was considered to indicate statistical significance.

Results

Estimation of platelet activation and function

Levels of P-selectin, as a measure of platelet activation, were higher in patients (median 24%, range 5·5–67%) than in controls (median 12%, range 6–23%, $P < 0·0004$), but did not correlate with platelet counts ($P = 0·08$). Twenty-one patients had increased levels (> 23%, the highest value in controls). Increased levels of P-selectin were not related to the need for treatment, as decided clinically, nor to any of the past treatment modalities. Shear-induced platelet adhesion was significantly lower in cAITP than in controls (median 2·4, range 0·1–17·4 vs. median 8·4, range 3·8–19·3; $P = 0·001$) since fewer platelets were available for adhesion in thrombocytopenia, as demonstrated by the correlation with platelet counts (Fig. 1a). Patients with increased levels of P-selectin had similar SC levels as those with normal P-selectin (median 2·4%, range 0·1–10·6% vs. median 3·6%, range 0·1–17·4%, $P = 0·5$) and thus the SC was not correlated with the expression of P-selectin ($r = 0·08$, $P = 0·6$; Fig. 1a). The AS, however, was not different in cAITP from controls (cAITP median 37, range 20–124 vs. controls 28, range 17–136; $P = 0·5$) and therefore not correlated with platelet counts (Fig. 1b). These results suggest that platelets in cAITP have an increased capability to form aggregates, compensating for the number of platelets that are available for aggregate formation. Individuals with elevated levels of P-selectin positive platelets had larger aggregates (median 34 μm^2, range 17–136 μm^2) compared to patients without elevated levels of P-selectin (median 25 μm^2, range 19–62 μm^2; $P = 0·03$; Fig. 1b), and the AS was correlated with the expression of P-selectin ($r = 0·4$, $P = 0·01$).

Platelet function and bleeding

The expression of P-selectin did not correlate with the bleeding score ($P > 0·05$). We next determined whether adhesion (SC) and aggregate size (AS) correlated with the bleeding score. As shown in Fig. 2 a there was a significant inverse correlation between the bleeding score and SC, while no correlation was seen with AS (Fig. 2b). As the risk for haemorrhage increases with low platelet counts we were interested to determine if the correlation of the SC with the bleeding score was only due to its correlation with platelet counts. We combined the subgroup of patients with no or only minor signs of bleeding (a score of 0 and 1) and compared these with patients with many petechiae and overt mucosal haemorrhage (a score of 2 or 3). Platelet counts (OR 0·378, 95%CI 0·16–0·65; $P = 0·0045$) and the SC (OR 0·27, 95%CI 0·095–0·55; $P = 0·0029$) were significantly associated with the bleeding score, while the size of aggregates was not (OR 0·932, 95%CI 0·85–0·98; $P = 0·057$). Platelet counts and the SC remained independently inversely correlated with bleeding symptoms as estimated

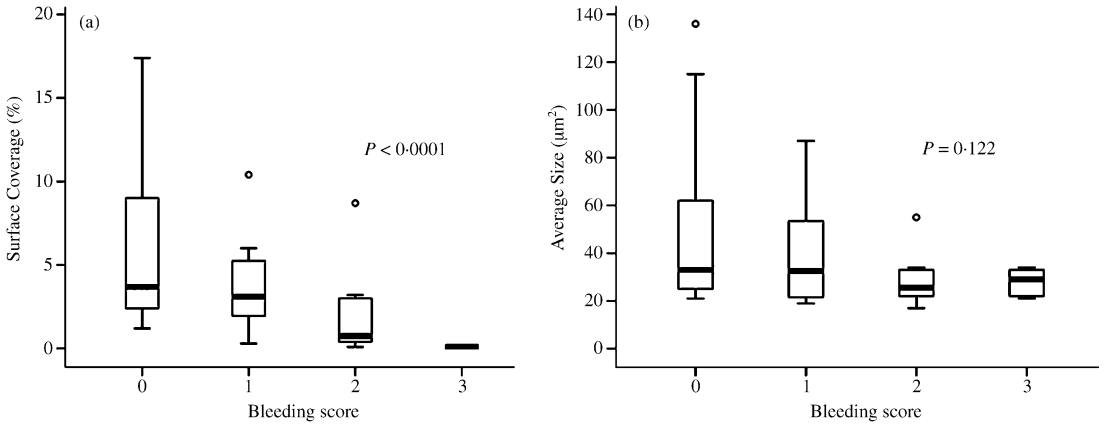

Figure 2 Clinical estimation of haemorrhage and high shear-induced platelet adhesion and size of aggregates. (a) Bleedings were scored 0–3 for no evidence of bleedings to overt mucosal bleedings and correlated with high shear-induced adhesion (Surface Coverage), and (b) the Average Size of the objects (Average Size) in 41 patients with chronic autoimmune thrombocytopenia. The median and upper and lower quartiles are shown in boxes with whiskers for the range of data and open symbols for outliners.

by multivariate analysis (platelet counts OR 0·5, 95%CI 0·22–0·88; $P = 0·04$; SC OR 0·45, 95%CI 0·17–0·87; $P = 0·04$).

The presence of detectable platelet antibodies (Table 1) was not correlated with platelet function or the bleeding score ($P > 0·05$; data not shown).

Discussion

The results showed that shear induced platelet adhesion correlates with the clinical estimation of haemorrhage in patients with cAITP. *In vivo* activated platelets, as determined by P-selectin expression, are particularly responsive to shear induced platelet aggregate formation.

The estimation of the risk for bleeding is mainly based on the platelet count, but some patients hardly bleed at even very low platelet numbers, while others have haemorrhage out of proportion even at 'save' platelet numbers of 50×10^9 L^{-1} platelets or more [1]. Platelet counts govern the need for treatment, however, as the *ex vivo* estimation of platelet function is limited due to their low numbers and the estimation of the risk for bleeding is subjective. An elevation of platelet counts is expected to reduce the risk of haemorrhage, but better knowledge of the platelet function can clearly influence the decision for sometimes harmful treatment [21–23].

Secured vascular integrity requires platelet activation, which can be estimated by the expression of P-selectin. In this study P-selectin did not correlate with platelet counts, supporting the assumption that the detectable expression of P-selectin can be influenced by various factors, like P-selectin positive platelets may not circulate but remain at sites of vascular injury and therefore not be available in the peripheral blood for their investigation. Further, levels of P-selectin expression were also not correlated with the clinical staging of haemorrhage. This finding may be due to rapid shedding of P-selectin, and platelets then appear P-selectin-negative, even though they still function [24]. It may also be that P-selectin positive platelets undergo premature sequestration as the latter is an important characteristic of cAITP. Thus, the estimation of P-selectin expression, even though often elevated in AITP patients, did not seem to aid the estimation of platelet function in many cAITP patients.

The correlation of the platelet adhesion with their counts is not surprising as the number of platelets determines how may are available to adhere to the polystyrene surface. Thus, it has been shown that the SC correlated with platelet counts in patients with a variety of thrombocytopenias [25]. In the former study an extracellular matrix rather than polystyrene was used for platelet adhesion. This material is more physiological than the current device, but still we show a significant inverse correlation of the SC with the clinical staging for haemorrhage, and this correlation was independent of platelet counts. A reduction of adhesion has been observed in samples that have been exposed to extracellular matrix, or von Willebrand factor, or collagen, the *in vivo* equivalent to proteins of the exposed subendothelium of injured vascular tissue [26]. We therefore may assume that at least part of the circulating platelets from cAITP patients were at some stage of activation as they had contact with the subendothelium, particularly in patients with haemorrhage, and therefore did not adhere well to the polystyrene surface, resulting in low SC. Further, platelets from Glanzmann thrombasthenia do not adhere [27] and those from Bernard Soulier show markedly reduced adhesion to the polystyrene surface [28], pointing at the importance of competent GPIIb/IIIa and GP Ib/IX. It may therefore be assumed that anti-GPIIb/IIIa and/or anti-GPb/IX autoantibodies, which were detected in 14 patients, modulated adhesion. This frequency of detectable platelet antibodies in patients with cAITP is within the range that has been reported in the past [29]. However, we may have not aimed for the detection of the relevant platelet

antibodies. This is unlikely, however, as GPIb/IX and GPIIb/IIIa are the most frequent targets for platelet antibodies in cAITP, and antibodies against other targets rarely occur without antibodies against these haemostatically very important platelet glycoproteins [5,30]. The number of individuals with these antibodies may have been too small to detect a correlation between the presence of these autoantibodies and adhesion. In rare cases platelet antibodies interfere with platelet function exposing the affected individuals at high risk for haemorrhage [6,8].

The size of aggregates was not related to platelet counts and, of note, it was not different from that in healthy individuals. The latter observation suggests that platelets in cAITP have an increased capability to form aggregates, compensating for the number of platelets that are available for aggregate formation. Platelets, which are activated and express P-selectin are likely to be involved in this process. This assumption is substantiated by our observation that patients with elevated levels of P-selectin positive platelets had formed larger aggregates upon the application of high shear than those without. The presence of P-selectin may indicate vesicle formation. As microparticles have strong procoagulatory activity their presence may explain the relatively mild bleeding in patients with cAITP.

In conclusion, the bleeding tendency varies considerably from one patient with chronic AITP to the next, but in many patients the bleeding tendency remains constant for weeks or even months. Confirmatory studies in larger patient populations are needed to determine if the combined estimation of adhesion and aggregate formation under high shear condition aid to assess the individual risk for haemorrhage to guide treatment.

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