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Short Communication

Recirculated normal platelets adhere to surfaces coated with plasma from patients with immune thrombocytopenia

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Immune thrombocytopenic purpura (ITP) patients have characteristic anti-platelet antibodies in their circulation. To assess the interaction between such antibodies adhering on to a non-physiological surface and human platelets, normal anticoagulated blood was perfused over ITP patient plasma-coated surfaces in a parallel plate flow chamber. At 300 s^{-1}, platelet adhesion to patient plasma-coated glass coverslips (24.0 ± 10%) was significantly higher than the adhesion to normal plasma-coated surfaces (9.8 ± 7%). When perfused at 1300 s^{-1}, the adhesion to patient plasma- (5.1 ± 1.3%) and to normal plasma- (2.5 ± 1.2%) coated coverslips were significantly weaker. Furthermore, patient platelet binding depended on simultaneous contributions by antibodies and fibrinogen present on the plasma-coated surface, since adherence was antagonized both by normal immunoglobulins added to the perfusate, as well as by the anti-GPIIb/IIIa monoclonal antibody 16N7C2, which competes with fibrinogen for binding to its receptor on the platelet. Accordingly, platelet adhesion was only observed to coverslips coated with the plasma but not the serum of ITP patients. Hence, perfusion of normal platelets over surfaces coated with ITP patient plasma enables a functional assessment of the presence in this plasma of anti-platelet antibodies.

Key words: Immune thrombocytopenia, shear-induced platelet adhesion, anti-platelet antibodies, immune suppression, glycoprotein IIb/IIIa.

Introduction

Idiopathic thrombocytopenic purpura (ITP) is a hemorrhagic disorder defined by a low platelet count and a reduced platelet survival, in association with a normal or increased number of megakaryocytes in the bone marrow.[1] The immune nature of this disorder, pointed out by Harrington and coworkers in 1951 is now well recognized.[2] Platelet associated antibodies are mostly directed against the platelet receptors GPIIb/IIIa, the GPIb–IX complex and less frequently against GPV or GPIa/IIa.[3,4] Anti-platelet antibodies not only enhance the turnover of platelets but may also cause their activation.[5]

Recent studies have shown that even the simple coating of microtiter plate surfaces with non-fractionated plasma sufficed to induce platelet adhesion to these surfaces.[6,7] This adhesion promoted under static conditions depends primarily on interactions between coated fibrinogen and platelet GPIIb/IIIa,[7,8] whereas immunoglobulins do not participate in the adhesion of resting platelets.[8]

Perfusion chambers first developed by Baumgartner and Sakariassen,[9,10] under flow conditions, allow the exploration of platelet interactions with surfaces containing insolubilized ligands. These studies have highlighted the importance of several platelet receptors, especially GPIb and GPIIb/IIIa in the adhesion of platelets to vessel wall components.

When plasma is allowed to coat on foreign surfaces, such as vascular protheses, platelets can attach and subsequently spread on these surfaces, thus

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initiating thrombus formation. In view of this adhesion, in the present study we have investigated how shear forces affect the interaction between platelets and surfaces coated with normal plasma or with ITP plasma proteins, amongst which anti-platelet membrane protein antibodies. Furthermore, we have used this platelet adhesion assay to *in vitro* investigate whether immunoglobulin addition to circulating blood would neutralize the antibody dependent platelet adhesion.

Patients and methods

Patient inclusion

Nine consecutive patients with ITP were included in the study after informed consent. The diagnosis was based on the following criteria: thrombocytopenia with a platelet count below 150×10^9/l, normal or increased number of bone marrow megakaryocytes, shortened platelet survival, absence of other causes of increased platelet destruction, and finally negative tests for lupus, hepatitis or HIV antibodies. Anti-platelet antibodies were not considered an inclusion criterion. Table 1 summarizes the clinical data for the patients included in the study.

Perfusion technique

A parallel plate perfusion chamber as previously described by Sakariassen *et al.*[10] was used to study the effect of immobilizing plasma or serum proteins from ITP patients on the degree of adhesion of platelets present in circulating normal whole blood. Ten ml blood from healthy volunteers not taking any medication compromising platelet function or coagulation during the week preceding the study, was collected by clean venipuncture into 0.1 vol 110 mM trisodium citrate. To avoid any incompatibility related to blood group type or Rhesus system, blood was only taken from normal subjects with blood group 0.

Platelet-poor plasma (PPP) was obtained by centrifugation of whole blood for 15 min at $3000 \times g$. Sera were prepared from non-anticoagulated whole blood after incubation at 37°C for 1 h. Supernatant sera were centrifuged and stored at −20°C until use. Perfusion was carried out at 37°C at a constant shear stress of 300 s^{-1} to mimic blood flow in the larger arterial vascular bed and at 1300 s^{-1} to reproduce the shear forces encountered in arterioles and stenotic areas.

Glass coverslips were coated overnight with 100 μl of serum or PPP in a humidified chamber. In order to investigate whether as a consequence of the Vroman effect,[11,12] a modification with time of the plasma protein composition on the coverslip would lead to alterations in the extent of platelet adherence, coverslips were coated for various time intervals (30 min to 20 h at 4°C). Normal or ITP serum or plasma-coated coverslips were then perfused and recirculated for 5 min with 10 ml of citrated whole blood derived from normal subjects (group O Rh^+) and prewarmed at 37°C for 5 min. Coverslips were removed from the perfusion chamber, rinsed in HEPES buffer and fixed with 1% glutaraldehyde in PBS prior to staining with May–Grünwald Giemsa.

The disappearance of single platelets (SPD) was calculated using the following formula: SPD = {(final platelet count in EDTA) − (final platelet count in Formol)}/(initial platelet count in EDTA). The platelet count of the blood samples was determined before and after each perfusion with a

Table 1. Patient characteristics of ITP patients included in the study

Patient number	Sex	Age (years)	Duration disease (months)	Platelet count (per μl) $\times 10^9$/l	Fibrinogen (g/l)	Treatment
1	M	65	1	71	1.97	G
2	M	23	48	8	3.77	c+S+Cy
3	F	24	1	22	3.21	–
4	F	45	156	9	2.9	c
5	F	74	2	116	2.29	c
6	F	41	< 1	2	2.0	–
7	F	42	120	118	1.75	G
8	F	21	132	80	3.46	c+S
9	F	21	16	25	1.58	C+G+S

G: Gammaglobulins; C: corticoids high dose (> 0.5 mg/kg); c: corticoids low dose; Cy: cyclosporin; S: splenectomy.

Cell-Dyn 610 (Sequoia Turner Corporation, Mountain View, CA).

Quantitation of platelet adhesion was done by analysis of the surface covered using *en face* light microscopy (Dialux 20 EB, E. Leitz GmbH, Wetzlar, Germany) at a low magnification (× 40)[9] and the 'TCL-Image' image processing software (Multihouse TSI, Amsterdam, the Netherlands).

Kinetics of fibrinogen and immunoglobulin coating

To investigate whether the amount of fibrinogen and immunoglobulin deposited on the glass coverslip would vary as a function of time, in agreement with the Vroman effect,[11,12] coverslips were coated in a humidified chamber with normal plasma (100 μl) and ITP patient plasma at 4°C for 30 min and for 2, 4, 6 and 20 h. The coated coverslips were then rinsed with 10 mM HEPES buffer, pH 7.35, containing 145 mM NaCl and non-occupied sites were saturated with 1% BSA dissolved in PBS, following a classical ELISA approach. Coverslips were then incubated overnight at 4°C with either goat-anti-human fibrinogen antibodies conjugated to horseradish peroxidase (HRP; Dakopatts) or with a monoclonal anti-human Ig antibody-HRP conjugate (Southern Biotechnology Ass., Inc., Birmingham, UK). Finally, the coverslips were rinsed and bound peroxidase was detected via staining with orthophenylenediamine and photometric detection at 492 nm.

Mechanism of platelet adhesion

In order to further assess the role of platelet GPIIb/IIIa in the adhesion of platelets to the coated surfaces, perfusions were performed in the presence of the monoclonal anti-GPIIb/IIIa antibody 16N7C2 (30 μg/ml) that competitively blocks fibrinogen binding to human GPIIb/IIIa.[13] This antibody was added to the perfusate 30 min prior to the perfusion.

In view of the postulated presence of anti-idiotypic antibodies in pooled human immunoglobulins, capable of neutralizing anti-platelet antibodies,[14] pooled lyophilized immunoglobulins (Ig Sandoglobulins, Basle, Switzerland) were dissolved in the anticoagulated blood to 6 mg/ml at room temperature for up to 1 h before perfusion over normal or ITP patient plasma-coated surfaces.

Results

Time-dependence of fibrinogen and immunoglobulin coating

A kinetic analysis of the amounts of fibrinogen and immunoglobulins adhering during the coating of coverslips with plasma, was suggestive of a time-dependent decrease of the levels of adhering immunoglobulins at short incubation times up to 4 h, followed by a sharp rise after 6 h. Although this increase was not significant in the case of the normal plasma immunoglobulin coatings, the patient plasma immunoglobulin peak after 6 h of coating, reached statistical significance ($P < 0.05$) when compared with the adhering immunoglobulin levels after overnight coatings (Table 2). The fibrinogen levels on the contrary fluctuated less (Table 2). To further investigate whether this change in protein composition would affect the degree of platelet adhesion, anticoagulated blood was perfused over coverslips coated with normal and ITP plasma for various time intervals. No significant differences were observed however between the surface coverages between coating times of 2, 4, 6, 8 and 20 h. Therefore, the remainder of the experiments were conducted with coverslips coated overnight at 4°C.

Table 2. Variation with time of the levels of fibrinogen and immunoglobulin binding to glass coverslips during the coating at 4°C of normal and patient plasma, analysed via peroxidase coupled antibody binding and light absorption measurements*

	Patient plasma		Normal plasma	
Time (h)	Fibrinogen (A_{492nm})	Immunoglobulin (A_{492nm})	Fibrinogen (A_{492nm})	Immunoglobulin (A_{492nm})
0.5	0.240 ± 0.142	0.769 ± 0.545	0.343 ± 0.063	0.494 ± 0.489
2	0.210 ± 0.038	0.689 ± 0.157	0.237 ± 0.129	0.460 ± 0.272
4	0.303 ± 0.179	0.554 ± 0.083	0.261 ± 0.173	0.339 ± 0.194
6	0.250 ± 0.089	2.002 ± 0.814	0.169 ± 0.017	0.644 ± 0.464
20	0.342 ± 0.234	0.374 ± 0.051	0.185 ± 0.152	0.292 ± 0.110

*Results are the mean ± SD for the binding of three normal and three patient plasmas.

Effect of shear forces on platelet adhesion

The perfusion at 300 s^{-1} of anticoagulated blood derived from normal donors over coverslips coated overnight with normal plasma, resulted in the weak adhesion of irregularly scattered platelets only, the adhesion being somewhat increased when plasma was used from a pool of healthy volunteers. Adhering platelets were found as single cells or as small aggregates consisting of a few platelets only (Figure 1a). Morphologically, these platelets were activated, as judged from their irregular shape and spreading. In contrast, when normal anticoagulated blood was perfused over coverslips coated overnight with plasma derived from ITP patients, a much stronger and more homogeneous platelet adhesion was observed (Figure 1b), with evidence of occasional platelet spreading but especially of the presence of large aggregates.

When perfusions were performed at 1300 s^{-1}, platelet adhesion to normal plasma-coated surfaces was reduced to a few single platelets (Figure 1c), whereas the perfusion over ITP plasma-coated surfaces, in addition to the presence of primarily single platelets, still resulted in the appearance of larger aggregates (Figure 1d).

Although large interindividual variations were observed for different patient plasmas, the mean platelet surface coverage calculated for perfusions at 300 s^{-1} over ITP patient plasmas was significantly increased (24.0 ± 10.4%) in comparison with the surface coverage of 9.8 ± 7% derived for perfusions over normal plasma ($P < 0.05$). However, when perfusions were performed over patient plasma-coated surfaces at 1300 s^{-1}, a significantly weaker platelet adhesion was observed (5.1 ± 1.3%). For perfusions over normal plasma coated surfaces, at 1300 s^{-1} even lower surface coverages were found (2.5 ± 1.2%).

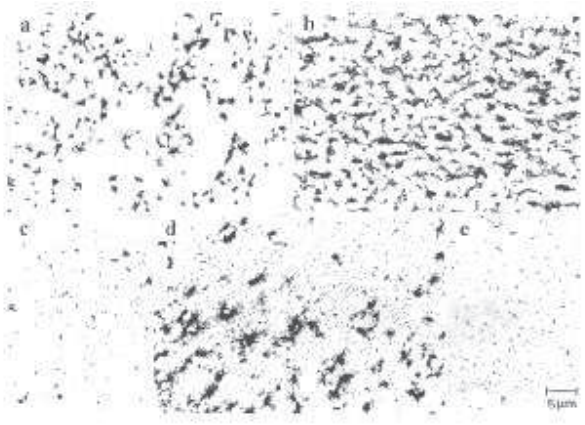

Figure 1. Platelet adhesion after perfusion of normal anticoagulated blood over glass coverslips coated with normal plasma (A, C), or ITP patient plasmas (B, D and E), perfused at 300 s^{-1} (A, B, E) or at 1300 s^{-1} (C, D), in the absence (A–D) or in the presence of antibody 16N7C2 (E). Scale bars represent 20 mm.

Platelet numbers present in the anticoagulated blood prior to perfusion ranged from 200 to 300 × 10^9/l. After the perfusion over the plasma-coated surfaces, these numbers slightly dropped. Calculation of the average SPD values for a total of 31 different runs yielded a mean index for the disappearance of platelets equal to 15.8 ± 8.3%.

Role of fibrinogen-GpIIb/IIIa axis in the adhesion

When perfusions were performed on surfaces coated with serum, no platelet adhesion was observed, neither for perfusions over ITP patient sera-coated surfaces nor for perfusions over normal serum-coated surfaces (0.10 ± 0.03%). Since these experiments suggested the involvement of coated fibrinogen, perfusions over plasma-coated surfaces were also performed in the presence of the anti-GpIIb/IIIa monoclonal antibody 16N7C2; this antibody (30 μg/ml) indeed blocked platelet adhesion completely when perfused at 300 s^{-1} over ITP patient-coated plasma, i.e. the surface coverage dropped from an average 16% in the absence of 16N7C2 to 0.1% in its presence (Figure 1e).

Neutralization of adhesion by added immunoglobulins

Pooled lyophilized human immunoglobulins dissolved in the perfusate to 6 mg/ml, had a small effect on the weak surface coverage observed during perfusions over normal plasma-coated coverslips, causing a non-significant drop from 3.7 ± 3.1 to 1.4 ± 1.4% ($n = 3$). However, immunoglobulin addition reduced the more prominent platelet surface coverage found during perfusions over ITP plasma-coated surfaces significantly from 13.1 ± 6.4% to 1.9 ± 0.97% ($n = 3$, $P < 0.05$). Immunoglobulin addition to the anticoagulated blood at higher concentrations (12 mg/ml) was associated with a drop in platelet numbers during the subsequent perfusion and was not further analyzed. Control experiments in which coverslips were directly coated overnight with gammaglobulins and perfused with normal anticoagulated blood did not show any platelet adhesion to such coverslips (not shown).

Discussion

The existence of anti-platelet antibodies in immune thrombocytopenic purpura patients is evident, but

the pathogenic role of such antibodies remains unclear. A good correlation was reported between the levels of platelet associated IgG and the clinical evolution or response to treatment in the acute and chronic ITP.[15,16] However, despite their very low platelet counts, ITP patients usually suffer few bleeding events which complicates an assessment of their bleeding risk.

In the present study, we used an *in vitro* perfusion system to functionally analyse the presence of anti-platelet antibodies in the plasma of ITP patients. We found that normal platelets consistently adhered to ITP plasma-coated coverslips to a higher degree than to normal plasma-coated surfaces suggestive of an anti-platelet antibody mediated adhesion of platelets to ITP plasma-coated surfaces. Even though variations of antibody binding to the coverslip were noted with time, these variations were not associated with a significant reduction in the degree of adhesion, however, the inhibition of platelet adhesion to ITP plasma-coated surfaces by immunoglobulins added to the perfusate nevertheless substantiates a role for anti-platelet antibodies during platelet recruitment. Indeed, it has been postulated that anti-idiotype antibodies present in pools of immunoglobulins may neutralize the anti-platelet antibodies[14] and hence reduce the adherence of circulating platelets.

However, since platelet adhesion was entirely lacking when ITP serum was used for the coating, the observed platelet adhesion was not exclusively mediated by anti-platelet antibodies but also depended on immobilized fibrinogen, a conclusion further supported by our finding that an antibody, antagonizing fibrinogen binding to its receptor on platelets (GPIIb/IIIa), completely prevented platelet adhesion to ITP plasma-coated coverslips. Thus, our findings confirm under conditions of flow that the adhesion of platelets to plasma protein-coated surfaces occurs via GPIIb/IIIa interactions with coated fibrinogen, a conclusion further supported by the observed platelet spreading, a typical property of platelet interactions with coated fibrinogen. Our data also show that the presence in the circulation of anti-platelet antigen antibodies aggravates platelet adhesion to foreign surfaces, further increasing the risk for platelet aggregation and thrombosis on such surfaces. These phenomena are more evident at shear forces encountered in the larger arteries (0.2–0.5 cm), i.e. in those arteries subject to vascular replacement surgery than at higher shear forces such as encountered in smaller arteries. The weaker interactions at high shear forces can be explained by the contribution to the platelet binding of the GPIIb/IIIa–fibrinogen interaction, an interaction which is not sufficiently strong to resist high shear forces.[17]

In conclusion, our data show that a brief exposure of flowing normal blood to coverslips coated with plasma but not with serum from ITP patients, induced antibody dependent platelet adhesion. Further investigations will be required to define whether the *in vitro* perfusion of normal platelets over ITP plasma-coated surfaces will constitute a valuable functional test to *ex vivo* assess the presence of anti-platelet antibodies in the plasma of ITP patients and to monitor their disappearance on treatment.

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