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Haematologica 2013 [Epub ahead of print]

doi:10.3324/haematol.2013.087874

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Patient autoantibodies induce platelet destruction signals via raft-associated glycoprotein Ibα and FcγRIIa in immune thrombocytopenia

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Running title: Autoantibody-induced platelet activation

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Acknowledgments

This study was supported by a grant from the Landsteiner Foundation of Blood transfusion Research (LSBR grant no. 0807). Prof. Dr. J.W.N. Akkerman is supported by the Netherlands Thrombosis Foundation. Dr. R.T. Urbanus is a research fellow of the Dutch Heart Foundation (grant no. 2010T068)
Immune thrombocytopenia (ITP) is an acquired immune-mediated disorder characterized by thrombocytopenia in the absence of an underlying cause.\textsuperscript{1} The pathophysiology of ITP is multifactorial and includes the development of autoantibodies that trigger abnormal thrombopoiesis, enhanced platelet destruction, complement activation and T-cell-mediated effects.\textsuperscript{1,3} Platelet autoantibodies are detected in about 50\% of patients\textsuperscript{4} and generally target the fibrinogen receptor $\alpha$IIb$\beta$3 or the receptor for von Willebrand factor (VWF), the glycoprotein (GP) Ib-V-IX complex. Anti-$\alpha$IIb$\beta$3 antibodies (70-80\% of cases) are thought to induce thrombocytopenia through increased platelet clearance by Fc$\gamma$ receptor-bearing macrophages. Autoantibodies against GPIb-V-IX (20-40\% of cases) often induce a more severe fall in platelet count\textsuperscript{5} that is less responsive to standard therapies, such as intravenous immunoglobulin G (IVIG).\textsuperscript{6} Thrombocytopenia induced by GPIb-V-IX autoantibodies has been characterized in little detail. Some monoclonal antibodies against GPIb$\alpha$ are known to induce platelet activation,\textsuperscript{7} which may lead to accelerated platelet destruction in ITP patients\textsuperscript{8} with autoantibodies against this receptor. Here we report how an autoantibody against GPIb$\alpha$, obtained from a patient with ITP, induces recognition signals for macrophages through interplay between glycoprotein Ib$\alpha$ and the low affinity IgG receptor Fc$\gamma$RIIa in lipid rafts.

The patient is a 70 year old Caucasian woman. She had a history of nephrectomy and parathyroidectomy; both surgeries were without bleeding complications. In 2007, routine laboratory investigations revealed a thrombocytopenia (20x10\textsuperscript{9}/L; normal 150-450x10\textsuperscript{9}/L). Recently, she suffered from increased spontaneous skin hematomas and melena. Her platelet count was low (20x10\textsuperscript{9}/L) and disturbed by aggregates. Hemoglobin level, leukocytes and differential were normal. There was no detectable monoclonal protein and immunoglobulin levels were normal. IVIG (30 g per day for 5 days) and platelet transfusions failed to increase platelet count to normal levels. Gastro- and colonoscopy did not reveal a clear bleeding focus (Supplementary Table S1). A detailed description of the materials and methods used is found in the Supplement.

Citrated blood from the ITP patient showed a mixture of single platelets and small aggregates. Inhibition of platelet activation with prostacyclin (PGI\textsubscript{2}) during blood collection reduced the number of aggregates, but the platelet count remained low (Figure 1A). FACS analysis with gating for single platelets showed increased levels of surface-expressed fibrinogen, P-selectin and PS compared with controls (Figure 1B). Surface-exposed P-selectin and PS are clearance signals, triggering platelet binding to macrophages, followed by their destruction.\textsuperscript{9} As expected, matured monocytic THP-1 cells phagocytosed 21-fold more patient platelets than control platelets. The activation observed in blood was caused by a plasma constituent, since normal platelets incubated in patient plasma were also activated and showed a strong increase in surface-bound fibrinogen and P-selectin. IgG depletion prevented the increase in P-selectin expression, which was restored upon repletion of IgG (Figure 1C).

The observed platelet activation suggested the presence of anti-GPIb$\alpha$ autoantibodies, as some monoclonal anti-GPIb$\alpha$ antibodies are known to induce platelet aggregation.\textsuperscript{7} Indeed, removal of
GPIbα ectodomain with o-sialoglycoprotein endopeptidase (osge) or addition of excess soluble recombinant GPIbα reduced P-selectin expression on normal platelets incubated in patient plasma (Figure 2A). Binding of VWF to GPIbα triggers its translocation to cholesterol-rich domains known as lipid rafts. Subsequent platelet activation by GPIbα involves signaling through immunoreceptor tyrosine-based activation motif-containing receptors, such as the Fc receptor γ chain (FcRγ) or the low-affinity IgG receptor FcγRIIa. To investigate whether a similar mechanism might function in autoantibody-induced platelet activation, suspensions of normal platelets in patient plasma were incubated with GM3 ganglioside, which inhibits GPIbα translocation to lipid rafts, or with a neutralizing anti-FcγRIIa antibody (clone AT10). Both treatments strongly suppressed P-selectin expression induced by patient plasma (Figure 2A). Antibody titer determination with immobilized recombinant GPIbα confirmed the presence of GPIbα autoantibodies in patient plasma (Figure 2B). Direct analysis of IgG binding in suspensions of normal platelets in patient plasma confirmed the expected interference by recombinant GPIbα, whereas GM3 and anti-FcγRIIa antibody had no effect (Figure 2C). These data demonstrate that antibody binding is primarily regulated through GPIbα.

FRET measurement using FLIM is a sensitive technique to analyze protein colocalization on the intact platelet membrane. Analysis of normal platelets revealed that little GPIbα was present in lipid rafts (Figure 2D). The receptor was dispersed over the surface and formed little associations with FcγRIIa. Incubation in patient plasma induced GPIbα translocation to rafts, the formation of GPIbα clusters and GPIbα association with FcγRIIa. Addition of GM3 not only induced the expected blockade of GPIbα translocation to rafts but also prevented formation of GPIbα clusters and GPIbα association with FcγRIIa. Collectively, these findings indicate that patient autoantibodies against GPIbα trigger surface expression of GPIbα clusters, P-selectin and PS, which are all ‘eat-me’ signals for macrophages. Crucial is the association with lipid rafts, both for formation of GPIbα clusters and activation of FcγRIIa, whose ligand-binding properties are enhanced by localization to rafts. The result is a more severe drop in platelet count compared to patients with anti-αIIbβ3 antibodies. The fact that αIIbβ3 does not translocate to rafts upon ligand binding may explain the inability of autoantibodies against this receptor to efficiently activate FcγRIIa to generate additional destruction signals. Future studies should reveal whether generation of destruction signals is similar in other ITP patients with autoantibodies against GPIbα. Confirmation of this mechanism in a larger population may open ways to explore the prevention of autoantibody-induced GPIbα association with FcγRIIa in lipid rafts as a possible therapy in ITP.
Authorship and disclosures
R.T.U and J.W.A designed research, interpreted results and wrote the paper. E.G. and D.E.W. designed and performed research, collected and analyzed data and wrote the paper. C.A.K. and D.J.H. performed research, collected and analyzed data, and interpreted results. A.H. and R.E.S. collected and analyzed data, participated in discussions and interpreted results. H.C.G. interpreted results and participated in discussions. H.D. provided essential reagents and participated in discussions. The authors declare no competing financial interests.

The online version of this article has a Supplementary Appendix
References


Figure legends

Figure 1. Platelet activation by patient autoantibodies. (A) Platelet aggregation during blood collection. Blood was collected in citrate supplemented with PGI2 (10 ng/mL) and FSC/SSC-scatter was analyzed by FACS in isolated control platelets (left panel) or patient platelets without and with PGI2 (middle and right panel). (B) Patient platelets are activated during blood collection. FACS analysis of activated αIIbβ3 (bound fibrinogen; Fg), surface P-selectin (P-sel) and PS exposure. CFMDA-labeled platelets were incubated with matured monocytic THP-1 cells and phagocytosis (phago) was determined. (C) Normal platelets were incubated (2 hours, 22 °C) with autologous (control plasma) or patient plasma and analyzed for bound fibrinogen and P-selectin expression. Depletion of IgGs from patient plasma (IgG depl) reduced surface P-selectin to control levels, while repletion (IgG repl) increased its expression. Results are expressed as ratio of normal platelets in patient plasma over control plasma. Data are means ± SEM (n=4).

Figure 2. Autoantibody binding to GPIbα leads to FcγRIIa-mediated platelet activation. (A) Normal platelets were incubated with autologous (control plasma) or patient plasma (2 hours, 22°C) and surface P-selectin expression was measured following removal of extracellular GPIbα (osge; 80 μg/mL), addition of recombinant GPIbα to patient plasma (rGPIbα; 50 μg/mL), GM3 (50 μM) to prevent GPIbα translocation to lipid rafts or a neutralizing anti-FcγRIIa antibody (clone AT10; 50 μg/mL). (B) Determination of GPIbα antibody titer in patient plasma by ELISA. Patient plasma was prediluted (1:10) and added to wells coated with or without recombinant GPIbα to determine IgG binding. (C) Determination of IgG binding to platelets by FACS analysis under conditions as described for (A). (D) FRET/FLIM analysis of GPIbα translocation to lipid rafts, clustering and co-localization with FcγRIIa of normal platelets incubated with patient plasma. Platelets were fixed with 2% paraformaldehyde and stained with 6B4-488, 6B4-594 (1 μg/mL), CTB-594 (5 μg/mL) or CD32-488 (1 μg/mL). The fluorescence lifetimes of the donor fluorophore (6B4-488 or AT10-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and subsequently used to calculate the FRET efficiency. Normal platelets incubated in autologous plasma have dispersed GPIbα receptors that do not co-localize with GM1, a marker for lipid rafts, or FcγRIIa. Platelet incubation in patient plasma triggers GPIbα translocation to rafts, leading to its clustering and association with FcγRIIa, which is prevented by addition of GM3. Data are means ± SEM (n=3).
Urbanus et al Figure 1

A

control

- PGI₂

patient

- PGI₂

+ PGI₂

B

patient platelets

C

normal platelets

ratios

(patient/control plts)

ratios

(patient/control plasma)

Fg

P-sel

PS

phago

ratios

(patient/control plasma)

Fg

P-sel

IgG depl

IgG repl
A. P-selectin expression

- Ratio of patient plasma to control plasma

B. GPIbα antibody titer

- OD₄₅₀ nm

C. IgG binding

- Ratio of patient plasma to control plasma

D. FRET efficiency (%)

- GM1
- GPIbα-GM1
- GPIbα
- GPIbα-FcγRIIa

*Urbanus et al Figure 2
Supplementary Methods

Materials and antibodies

We used the following products (with sources): 5-chloromethyl-fluorescein-diacetate (Cell tracker green/CFMDA, Molecular Probes/Invitrogen, Carlsbad, CA), 96-well microtiter plate (Nunc Nickelate; Nalge Nune International, Rochester NY), anti-FcγRIIa neutralizing antibody (clone AT10; Sanbio, Uden, The Netherlands), bovine serum albumin (BSA) fraction V and vitamin D₃ (Sigma-Aldrich, St. Louis, MO), monosialoganglioside GM3 (GenWay Biotech Inc, San Diego, CA), EDTA-K2 (BD, San Diego, CA), fibrinogen (Enzyme Research Laboratories, South Bend, IN), O-sialoglycoprotein-endopeptidase (osge, Cederlane Laboratories, Hornby, Canada), phorbol-12-myristate-13-acetate (PMA, MP Biochemicals, Illkirch, France), platelet-derived human TGF-β1 (R&D systems, Minneapolis, MN), prostacyclin (PGI₂, Cayman Chemical, Ann Arbor, MI), Protein G-sepharose (GE Healthcare, Uppsala, Sweden), recombinant GPIba (high sulphated), sodium-heparin (Greiner bio-one, Frickenhausen, Germany), and THP-1 monocytic cells (ATCC/LGC Standards, Wesel, Germany).

For FACS analysis we used antibodies against human IgG (Fab-specific; Sigma-Aldrich), P-selectin and FITC-labeled fibrinogen (BD, San Diego, CA). Surface exposure of phosphatidylserine (PS) was deduced from FITC-labeled lactadherin binding (Haematologic Technologies Inc. Essex Junction, VT). For ELISA we used HRP-conjugated goat-anti-human IgG (Surmodics, Eden Prairie, MN). The agents used for FRET analysis were the recombinant Alexa Fluor-488 and -594-conjugated 6B4-Fab fragments (6B4-488 and 6B4-594, respectively) directed against aa 200-268 of GPIba,²³ Alexa Fluor-488-conjugated anti-FcγRIIa (AT10-488; Santa Cruz, biotechnology, Santa Cruz, CA), and Alexa Fluor-594-conjugated Cholera toxin subunit B directed against ganglioside GM1 (CTB-594; Invitrogen, Carlsbad, CA).

Blood collection, platelet isolation and incubations

Procedures were approved by the Medical Ethical Committee of our hospital. Platelets from healthy, medication-free volunteers were isolated⁴ and resuspended in Heps-Tyrole (HT) buffer (pH 7.3). Whole blood and isolated platelets were analyzed using the Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA). The patient platelets aggregated spontaneously during blood collection in vacuum tubes containing citrate, coated EDTA-K2 or sodium-heparin, preventing investigations. To reduce aggregation to a minimum, patient blood was collected by free-flow; the first 2 ml was discarded and the remainder collected in 13 mM citrate (f.c.), supplemented with 10 ng/mL PGI₂ to induce transient platelet inhibition. Patient platelets were subsequently isolated as described.⁴ Plasma was isolated by centrifugation (10 minutes, 2000g, 22 °C). For plasma reconstitution experiments, platelet-rich plasma from healthy subjects was mixed with 0.1 volume of ACD (2.5% trisodium citrate, 1.5% citric acid and 2% D-Glucose), centrifuged (15 minutes, 330g, no brake,
22 °C) and platelets were incubated in autologous or patient plasma for 2 hours, 22 °C. Then, platelets were washed (15 minutes, 330 g, no brake, 22 °C, in the presence of 10 ng/mL PGI₂), resuspended in HT buffer (pH 7.3) (2x10¹¹ cells/L) and kept at 22 °C for 30 minutes to regain responsiveness. In some experiments, platelets were incubated (30 minutes at 22 °C) with osge (80 µg/mL) prior to plasma incubation to remove GP Ibα ectodomain, with recombinant GP Ibα to saturate GP Ibα binding properties in patient antibody (50 µg/mL), with a neutralizing antibody against FcγRIIa (50 µg/mL) and with GM3 (50 µM) to interfere with GP Ibα translocation to lipid rafts.³

**Flow cytometric analysis**

Characterization of platelets by Fluorescence-Activated Cell Sorting (FACS) was based on forward- and side-scatter (FSC/SSC) (FACS-Calibur; BD Biosciences, San Jose, CA). Platelets were incubated with appropriate antibodies (15 minutes, 37 °C) and 10,000 platelets were analyzed for surface expression of P-selectin, phosphatidylserine (PS), fibrinogen and binding of human IgGs. Results were expressed as ratio of patient data/control data.

**Plasma depletion from IgGs**

Patient plasma and plasma pooled from 200 healthy donors was depleted from IgG by passage through a Protein G-sepharose column. The effluent was collected and stored at -80 °C before experiments. Bound IgG was eluted and dialyzed thrice against a large excess of 10 mM Hepes, 154 mM NaCl, pH 7.4 for 6 hours. IgG was stored at -80°C until used for repletion studies.

**Antibody titer determination by ELISA**

Antibody titer of GP Ibα antibodies was determined by ELISA. Wells were coated with 10 µg/mL of fully sulphated recombinant GP Ibα (residues 1-298),¹ blocked with 4% BSA and washed. Patient plasma was prediluted (1:10) and added in a 1:2 (vol:vol) dilution series. Bound antibody was detected with HRP-conjugated goat-anti-human IgG and quantified at 450 nm using a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA).

**Platelet phagocytosis**

Platelets were resuspended in HT buffer (pH 6.5) to a concentration of 4x10¹¹ cells/L, labeled with CFMDA (20 µM, 1 hour, 22 °C), centrifuged (330 g, 10 minutes, 22 °C) in the presence of 10 ng/mL PGI₂ and resuspended in HT buffer (pH 7.3). Functionality was recovered by 30 minutes incubation at 22°C. For measurement of phagocytosis, monocytic THP-1 cells were cultured in a 96-wells plate (5x10⁶ cells/well) and stimulated with 50 nM vitamin D₃ and 1 ng/mL TGF-β1 (12 hours, 37°C) and 250 nM PMA (2 hours, 37°C). CaCl₂ and MgCl₂ (1mM each, f.c.) were added and 5x10⁵ platelets were incubated with the maturated THP-1 cells (90 minutes, 37°C). Unbound platelets were removed and phagocytosis was measured on a Fluorstar Galaxy (BMG LABTECH GmbH, Offenburg, Germany). Results were expressed as ratio of patient data/control data.
Analysis of GPIbα distribution by Förster Resonance Energy Transfer using Fluorescence Lifetime Imaging Microscopy (FRET/FLIM)

GPIbα distribution by FRET/FLIM was analyzed as described before. In short, 6B4-Fab fragments conjugated to either Alexa Fluor-488 or Alexa Fluor-594 (6B4-488 and 6B4-594 respectively) were incubated with fixed platelet samples under conditions in which each Fab labeled ~50% of total receptor number. GPIbα translocation to lipid rafts was determined by labeling GPIbα with 6B4-488 and the lipid rafts marker GM1 with CTB-594 (5 μg/mL). GPIbα colocalization with FcγRIIa was analyzed by labeling FcγRIIa with AT10-488 (1 μg/mL) and GPIbα with 6B4-594. The fluorescence lifetimes of the donor fluorophore (6B4-488 or AT10-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and subsequently used to calculate the FRET efficiency, defined as

\[
\text{FRET Efficiency} = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100%
\]

where \(\tau\) is the donor fluorophore lifetime in nanoseconds in the absence (\(\tau_D\)) and presence (\(\tau_{D/A}\)) of the acceptor fluorophore.

Statistical analysis

Data are means ± SEM with number of experiments (n), as indicated. Statistical analysis was performed using GraphPad Prism 5 (San Diego, CA) software. Differences between control platelets and incubations were analyzed by Mann-Whitney test. \(P\)-values <0.05 (*) were considered significant.
**Supplementary Table S1: Hematology parameters**

Coagulation assays were performed on a Sta-rack coagulation analyzer (Diagnostics Stago, Asnieres, France).

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<th>Patient</th>
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