Platelet autoantibody-induced platelet clearance represents a major pathomechanism in immune thrombocytopenia (ITP). There is growing evidence for clinical differences between anti-glycoprotein IIb/IIIa and anti-glycoprotein Ib/IX mediated ITP. Glycoprotein V is a well characterized target antigen in Varicella-associated and drug-induced thrombocytopenia. We conducted a systematic study assessing the prevalence and functional capacity of autoantibodies against glycoprotein V. A total of 1140 patients were included. In one-third of patients, platelet-bound autoantibodies against glycoproteins Ib/IX, IIb/IIIa, or V were detected in a monoclonal antibody immobilization of platelet antigen assay; platelet-bound autoanti-glycoprotein V was present in the majority of samples (222 out of 343, 64.7%). Investigation of patient sera revealed the presence of free autoantibodies against glycoprotein V in 13.5% of these patients by an indirect monoclonal antibody immobilization of platelet antigen assay, but in 39.6% by surface plasmon resonance technology. These antibodies showed significantly lower avidity (association/dissociation ratio 0.32±0.13 vs. 0.73±0.14; P<0.001). High- and low-avidity antibodies induced comparable amounts of platelet uptake in a phagocytosis assay using CD14+ positively-selected human macrophages [mean phagocytic index, 6.81 (range, 4.75-9.86) vs. 6.01 (range, 5.00-6.98); P=0.954]. In a NOD/SCID mouse model, IgG prepared from both types of anti-glycoprotein V autoantibodies eliminated human platelets with no detectable difference between the groups from the murine circulation [mean platelet survival at 300 minutes, 40% (range, 27-55) vs. 35% (16-46); P=0.025]. Our data establish glycoprotein V as a relevant immune target in immune thrombocytopenia. We would suggest that further studies including glycoprotein V will be required before ITP treatment can be tailored according to platelet autoantibody specificity.
vided some evidence that autoantibodies may also trigger more complex processes, such as platelet activation, platelet desialylation, or platelet apoptosis, all of which could lead to Fc-independent platelet clearance. More recently, there has also been evidence that the glycoprotein specificity of the autoantibodies could be important; for example, in a study by Li et al., desialylation occurred in the presence of anti-GP Ib/IX, but not in the presence of anti-GP IIb/IIIa antibodies.

In general, antibody identification (or the use of monoclonal antibodies in animal models) in these studies was restricted to two types of autoantibody specificities: anti-GP IIb/IIIa and anti-GP Ib/IX. This was because these two glycoproteins are currently considered to be the most important autoimmune targets in ITP. However, glycoprotein V (GP V) is a major protein on the platelet membrane, with approximately 10,000 copies per platelet. More than 50 years ago, GP V was first thought to be the immune target of quinine-related platelet antibodies by Stricker and Shulman, and Garner et al. described GP V as the antigen in a gold-triggered autoimmune response in patients with rheumatoid arthritis. GP V was also described as the target protein in pediatric varicella-associated thrombocytopenia. Some evidence for a potential role of GP V in ITP came from preliminary studies in patients with different types of thrombocytopenia. A valuable systematic study on GP V in patients with ITP was recently published, but whether or not anti-GP V autoantibodies contribute to thrombocytopenia in ITP remains unknown.

Here, we investigated the potential of anti-GP V autoantibodies in patients with ITP. Our work shows that autoantibodies to GP V are found in a majority of patients with ITP and can potentially cause platelet clearance mechanisms. This new information helps fill in some of the missing pathophysiological events in ITP.

**Methods**

Adult patients with a suspected diagnosis of ITP were identified, as previously described. In brief, standardized questionnaires covering relevant criteria to refute or confirm a diagnosis of ITP according to the British guidelines were used. Patients with relevant other diagnoses that could explain thrombocytopenia, such as aplastic anemia, leukemia, lymphoma, myelodysplastic syndrome, solid tumors, liver cirrhosis, recent cardiac surgery, BM/blood stem cell transplantation, sepsis, and drug-induced thrombocytopenia, were not included. Platelet-bound and free anti-platelet autoantibodies of the IgG type were detected by the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay, as described by Kiefel et al. Assay sensitivity was controlled by the use of the anti-HPA-1a World Health Organization (WHO) standard (NIBSC, Potters Bar, UK).

Leftover material was used for the additional experiments performed. All anti-GP V sera used in these experiments were negative for the presence of anti-GP IIb/IIIa, anti-GP Ib/IX, anti-GP Ia/IIa, and anti-GP IV by MAIPA. Immunoglobulin G (IgG) fractions were isolated using a commercial purification kit (MelonTM-Gel IgG Spin Purification Kit, Thermo Fisher Scientific, Waltham, MA, US).

Surface plasmon resonance (SPR) analysis allows label-free, realtime investigation of antigen-antibody interactions. This was performed on a protein interaction array system (ProteOn XPR36, Bio-Rad, Munich, Germany). Recombinant His-tagged GP V as the target protein and GP IV as an irrelevant control (R&D Systems; Life Technologies, Carlsbad, CA, USA) were immobilized onto flow cells of an HTS sensor chip. Phosphate buffered saline-tween (PBS-T) was used as running buffer for all steps. The SPR signal originates from changes in the refractive index at the chip’s surface. For antigen-antibody interactions, changes in the refractive index are linear to the number of antibodies bound. Data were acquired with the computer software (ProteOn Manager Software, BioRad). Interaction curves were referenced by interspot, second flow cell with immobilized GP IV and monoclonal anti-GP V (MAAB42, R&D Systems, 6 μg/mL) as standard. The R700/R350 ratio was used to differentiate high-avidity (>0.5) and low-avidity (<0.5) antibody binding.

A phagocytosis assay was performed using CD14 positive-selected macrophages (autoMACS Pro Separator; Miltenyi Biotec, Germany) from cryogenically stored human spleen specimens obtained from ITP patients. Healthy donor platelets were fluorescently labeled with CellTracker Green 5-chloromethylfluorescein diacetate (Thermo Fisher Scientific, MA, USA), washed, then opsonized with the ITP serum samples and added to the splenic macrophages for phagocytosis. Macrophages were observed by spinning-disc confocal microscopy under 63x objective immersion with differential interference contrast (DIC) and laser fluorescence (488, 647 excitation) on a Quorum multi-modal imaging system (Quorum Technologies, ON, Canada) equipped with a 50 micrometer pinhole spinning disc and an ORCA-Flash 4.0 V2 sCMOS camera. Four images were taken at the center of each well with Z-stacking every 0.83 μm with >30 stacks. Images were reconstructed in 3D for analysis using Imaris 8.0.2 (Bitplane, UK) and phagocytic index was calculated as (total engulfed platelets / splenic macrophages counted) x 100.

A NOD/SCID mouse model was used to investigate the elimination of human platelets by anti-GPV autoantibodies in ITP patients. In brief, NOD/SCID mice (NOD.CB17-Prkdcscid/J; Stock No. complexes, 001303) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) via Charles River, Research Models and Services (Sulzfeld, Germany). Sex- and age-matched (8-16-week old) animals were used in this study. Human platelets (200 μL, 2x10^9/mL) were injected into the lateral mouse tail vein. After 30 minutes (min) a blood sample was collected by tail vein puncture to determine the baseline of circulating human platelets (100%). Subsequently, IgG fractions isolated from human sera containing anti-GPV antibodies or control sera from healthy donors were injected into the other lateral tail vein (2 mg/g body weight). The survival of human platelets in the mouse circulation was analyzed over time using flow cytometry (Cytomics FC 500; Beckman Coulter) after staining platelets with anti-human CD41-PE-Cy5 (Beckman Coulter) and anti-mouse CD41-FITC (BD Biosciences, San Diego, CA, USA). Animal experiments were performed with the approval of the local authorities inTuebingen, Germany. The study was conducted in accordance with the Declaration of Helsinki, and the use of human material was approved by the local ethics committees in Giessen, Germany and Toronto, ON, Canada.

**Results**

**Prevalence of platelet-bound autoantibodies against GP V**

A total of 1645 patients with no alternative reason for a low platelet count were included. The amount of autologous platelets was sufficient for a complete direct test (including all 3 glycoprotein specificities) in 1140 patients (69.3% of n=1645 patients with a clinical suspicion of
ITP. This group was further assessed in order to ensure comparability of data. Results are summarized in Table 1. For patients with a positive test result for at least one glycoprotein, the frequency of immunization against GP V was similar to the other glycoproteins: 242 out of 343 (70.6%) patients were positive for anti-GP Ib/IX, 232 out of 343 (67.6%) patients were positive for anti-GP Ib/IX, and 222 out of 343 (64.7%) patients were positive for anti-GP V (Kruskal-Wallis test; P=0.67) (Table 1).

Interestingly, there was also no difference in the amount of antibodies attached to GP V (antibody load), as determined by the optical density of the MAIPA assay between glycoproteins: mean values were 1.86 [95% confidence interval (CI): 1.49-2.23] for anti-GP Ib/IIa, 1.63 (1.27-1.99) for anti-GP Ib/IX, and 1.82 (1.37-2.26) for anti-GP V; Kruskal-Wallis test, P=0.77.

Prevalence and binding properties of free autoantibodies against GP V

Sera from patients with any positive result in the direct MAIPA test (n=343) were further assessed by indirect MAIPA for the presence of free autoantibodies against these platelet glycoproteins. Results are summarized in Table 1. Free autoantibodies were detected in 45 out of 343 (13.1%) patient samples. The glycoprotein-specific distribution was GP Ib/IIa (25 out of 45, 55.5%), GP Ib/IX (30 out of 45, 66.6%), and GP V (29 out of 45, 64.4%). Identified free autoantibody specificities matched the platelet-bound specificities from the same patient throughout. Addition of recombinant GP V to sera prior to testing completely blocked the detection of anti-GP V autoantibodies, but did not interfere with the detection of anti-GP Ib/IIa or anti-GP Ib/IX autoantibodies (data not shown).

Table 1. Summary of autoantibody specificities detected in 343 of 1140 immune thrombocytopenia patients, either on the surface of the patient’s platelets (platelet-bound) or free in patient serum.

<table>
<thead>
<tr>
<th>Glycoprotein specificity</th>
<th>Platelet-bound autoantibodies</th>
<th>Free autoantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N. of positive samples</td>
<td>%</td>
</tr>
<tr>
<td>GP Ib/IIa only</td>
<td>71</td>
<td>20.7</td>
</tr>
<tr>
<td>GP Ib/IX only</td>
<td>30</td>
<td>8.8</td>
</tr>
<tr>
<td>GP V only</td>
<td>10</td>
<td>2.9</td>
</tr>
<tr>
<td>GP Ib/IIa plus GP Ib/IX</td>
<td>20</td>
<td>5.8</td>
</tr>
<tr>
<td>GP Ib/IIa plus GP V</td>
<td>10</td>
<td>2.9</td>
</tr>
<tr>
<td>GP Ib/IX plus GP V</td>
<td>61</td>
<td>17.8</td>
</tr>
<tr>
<td>GP Ib/IIa plus GP Ib/IX plus GP V</td>
<td>141</td>
<td>41.1</td>
</tr>
<tr>
<td>Total</td>
<td>343</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Rounding error

Autoantibody-triggered phagocytosis and in vivo platelet clearance

Anti-GP V autoantibodies were grouped according to their SPR binding profiles into a “high avidity” and a “low avidity” group. IgG fractions prepared from two high-avidity and two low-avidity anti-GP V antibody-containing ITP sera were tested in a phagocytosis assay using CD14 positively-selected human macrophages from ITP spleens (Figure 2). One high- and one low-avidity GP V sera induced significant platelet uptake relative to normal human serum controls (P=0.008 and P=0.026, respectively). Of those positive, high- and low-avidity antibodies induced similar amounts of platelet uptake [mean phagocytic index, 6.81 (range, 4.75-9.86) vs. 6.01 (range, 5.00-6.98), respectively; P=0.954]. To further assess the biological effect of anti-GP V autoantibodies on platelet destruction, the NOD/SCID mouse model was used. First, moab SW16 against human GP V was injected at two concentrations and the results verified against a murine monoclonal antibody (SZ21) specific for GPIIb/IIIa known to cause thrombocytopenia. SW16 induced similar clearance of human platelets from the murine circulation as SZ21 (mean platelet survival after 300 min, 16±5% vs. 8±8%; P=0.140) (Figure 3A). Platelet elimination was slower when SW16 was injected at a lower concentration (27±4%; P=0.018) (Figure 3A). Next, we analyzed IgG fractions isolated from ITP sera which contained anti-GP V autoantibodies only. Unexpectedly, anti-GP V reduced the survival of human platelets compared to control IgG regardless of their binding properties [median platelet survival after 300 min, “high avidity”, 35% (range, 16-46%; P=0.029) and “low avidity”: 40% (range, 27-55%; P=0.025), respectively] (Figure 3B). After 24 h, only a few injected human platelet circulated in the presence of anti-GP V antibodies [median platelet survival after 1440 min, “high avidity”, 22% (range, 11-23%; P=0.026) and “low avidity”, 20% (range, 13-24%; P=0.029) vs. 46% (range, 43-76%) (Figure 3B). No difference in platelet elimination was observed between the two groups (P=0.229 and P=0.441, after 300 min and 1440 min, respectively). As expected, autoantibodies were generally less effective in panel]. These results indicate that SPR has better sensitivity compared to the gold standard MAIPA assay in detecting anti-GP V autoantibodies.

Glycoprotein V in ITP
removing platelets from the murine circulation than human alloantibodies (anti-HPA-1a as present in the WHO standard). These data demonstrate that anti-GP V antibodies of high or low avidity are capable of removing circulating platelets and thus represent a functionally relevant specificity of autoantibodies in ITP. To further substantiate the hypothesis that the observed effects are mediated by anti-GP V IgG, we directly compared the median human platelet survival after injection of IgG fractions prepared from one ITP serum containing anti-GP V autoantibodies only (Figure 3C), either after the absorption with recombinant glycoprotein V (rGPV; dashed line) or without (full line). The median platelet survival at t=1440 min after absorption was 48.5% (range, 44-53%) versus 18% (range, 11-20%) without absorption (P=0.028). This experiment supports our conclusion that anti-GP V IgG is capable of removing human platelets from the murine circulation.

Discussion

In this study, we demonstrate that GP V is a frequent immune target in ITP patients. Anti-GP V autoantibodies are detectable with the same frequency as those against GP Ib/IIa and GP Ib/IX. Anti-GP V autoantibodies have the ability to induce a modest level of phagocytosis and to eliminate human platelets in a murine model.
Autoantibodies against platelets are considered to be the major factors for platelet clearance in ITP. GP IIb/IIIa and GP Ibα are generally reported as the most common antigenic targets. We were able to analyze the amount of platelet-bound, glycoprotein-specific autoantibodies in 343 patients in parallel. Whereas anti-GP V antibodies as the sole autoantibody entity were less often detected (2.9%) than the other specificities (20.7% and 8.9%, respectively), two-thirds of all ITP patients reacted with GP V in combination with other specificities. Anti-GP V was more often seen in association with anti-GP Ib/IX (61 out of 91, 67%) than with anti-GP IIb/IIIa (10 out of 8, 12%), but all entities were clearly separable. In contrast to platelet-bound autoantibodies, free autoantibodies in patient plasma are only rarely detectable. Still, in our cohort, free anti-GP V was not less often detected than the other specificities, again, most frequently in association with other autoantibodies. Adding anti-GP V detection to the standard laboratory test would only mildly increase the overall test sensitivity (from 29.2% to 30.1% for platelet-bound glycoprotein specific autoantibodies and from 3.4% to 3.9% for free autoantibodies). In contrast to conventional testing by MAIPA, SPR technology significantly raised the test sensitivity, with no loss of specificity. Interestingly, we observed a clear difference in autoantibody avidity between those autoantibodies detected by standard serology plus SPR and those detected by SPR only. To our knowledge, autoantibodies against platelets have not been investigated for their avidity before. However, we previously demonstrated that anti-HPA-1a alloantibodies against platelets may be of low avidity and escape detection by MAIPA. These antibodies had a comparable profile to the SPR-only autoantibodies detected in this study: a slow binding during the association and fast detachment during the dissociation phase. This suggests that these antibodies may become washed away in conventional test methods, whereas no-wash detection by SPR increases sensitivity. Low-avidity anti-GP V autoantibodies were able to induce platelet destruction in vitro and in vivo. This finding indicates that these antibodies, which are not detectable using conventional methods, are of clinical relevance. This observation demonstrates that low sensitivity could, in fact, be an important drawback of autoantibody testing in the laboratory. Further development of methods might be useful to increase the clinical utility of platelet autoantibody testing.

Anti-GP V autoantibodies were efficient in removing platelets, regardless of their avidity, indicating that platelets loaded with anti-GP V undergo the same fate as platelets loaded with other autoantibodies. The presence of anti-GP V might affect the clinical picture of ITP patients in two ways: 1) by a more efficient platelet removal because of an increased overall IgG load; or 2) by
functional effects of anti-GP V with subsequent changes in platelet reactivity. Since this was a laboratory-based study with one-stage clinical and laboratory data only, and no follow up, no definite conclusions can be drawn.

The low level of phagocytosis induced by these autoantibodies may hint at a unique mechanism of thrombocytopenia, or could indicate that a co-factor found in vivo but not in vitro (complement components, C-reactive protein, or serum amyloid A) is required.30 Alternatively, it is possible that the highest affinity antibodies remain bound to platelets and those in the sera have lower affinity and, therefore, trigger lower levels of phagocytosis.

Antibodies against GP V could exert different functional effects on platelets: GP V is cleaved by thrombin or, following platelet activation with collagen, by ADAM17/TACE.31,32 GP V is thought to function as a negative modulator of thrombin-induced platelet activation.33 In vivo studies in mice have demonstrated that the absence of GP V increases both platelet adhesion and aggregation; but also decreases thrombus stabilization.13 Whether any of these physiological processes are affected by anti-GP V autoantibodies is currently not known. Since we have now established GP V as an important immune target in ITP, it will be important to study whether the presence (or absence) of anti-GP V antibodies also affects treatment efficacy, as previously reported for the two other autoantibody specificities.35,36

This study has some limitations. Only ITP patients in whom a complete direct MAIPA test could be performed qualified. This cohort may not be representative for all ITP patients. In addition, antibodies of the IgA or IgM type, which are rarely detected in ITP,27,28,37 were not studied. We were also unable to characterize IgG subclasses in our cohort. Whereas others have shown that the majority of anti-GPllb/IIIa autoantibodies are of the IgG1 subclass, some IgG2, 3 and 4 have been reported.38 The IgG subclass distribution of anti-GP V may differ from anti-GPllb/IIIa. Finally, any blood sample taken from an ITP patient may not reflect the in vivo situation, since platelets sensitized with high-avidity antibodies may have been cleared (together with these antibodies) from the circulation before the sample was taken.

Despite these restrictions, we have confirmed GP V as a frequent immune target in ITP and demonstrated that anti-GP V autoantibodies are of clinical relevance since they can remove platelets from the circulation. We have also, for the first time, demonstrated that low platelet autoantibody avidity might be the main reason why current serology does not detect platelet autoantibodies more often. We would suggest that studies including GP V as an immune target are required before ITP treatment can be tailored according to platelet autoantibody specificities.

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