Glycosylation of autoantibodies: Insights into the mechanisms of immune thrombocytopenia

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Summary
Immune thrombocytopenia (ITP) is a bleeding disorder caused by IgG autoantibodies (AAbs) directed against platelets (PLTs). IgG effector functions depend on their Fc-constant region which undergoes post-translational glycosylation. We investigated the role of Asn279-linked N-glycan of AAbs in vitro and in vivo. AAbs were purified from ITP patients (n=15) and N-glycans were enzymatically cleaved by endoglycosidase F. The effects of native AAbs and deglycosylated AAbs were compared in vitro on enhancement of phagocytosis of platelets by monocytes and complement fixation and activation applying flow cytometry, laser scanning microscopy, and a complement consumption assay. AAB-induced platelet phagocytosis was inhibited by N-glycan cleavage (median phagocytic activity: 8% vs 0.8%, p=0.004). Seven out of 15 native AAbs bound C1q and activated complement. N-glycan cleavage significantly reduced both effects. In vivo survival of human PLTs was assessed after co-transfusion with native or N-glycan cleaved AAbs in a NOD/SCID mouse model. Injection of AAbs resulted in rapid clearance of human platelets compared to control (platelet clearance after 5h (CL₅₅) 75% vs 30%, p<0.001). AAbs that were able to activate complement induced more pronounced platelet clearance in the presence of complement compared to the clearance in the absence of complement (CL₅₅ 82% vs 62%, p=0.003). AAbs lost their ability to destroy platelets in vivo after deglycosylation (CL₅₅ 42%, p<0.001). N-glycosylation of human ITP AAbs appears to be required for platelet phagocytosis and complement activation, reducing platelet survival in vivo. Posttranslational modification of AAbs may constitute an important determinant for the clinical manifestation of ITP.

Keywords
Thrombocytopenia, autoantibody, ITP, animal model, complement

Introduction
Immune thrombocytopenia (ITP) is a chronic autoimmune disease (1, 2), in which increased platelet destruction and ineffective thrombopoiesis result in low platelet counts (1-3). While direct cellular destruction of platelets and megakaryocytes may contribute to some extent to thrombocytopenia, platelet autoantibodies (AAbs) play likely a more important role (4-9). These AAbs are commonly directed against glycoproteins (GPs) on the platelet membrane, GPIIb/IIIa and GPIb/IX (3, 4, 10, 11). AAbs can induce platelet destruction by several mechanisms. Some of them mediate phagocytosis of antibody-coated platelets; others activate complement, which results in intravascular lysis of platelets (12, 13); and a subgroup of these AAbs in addition may interfere with platelet production (5).

Phagocytosis of AAbs opsonised platelets as well as complement activation are mediated by the Fc-portion of platelet-bound AAbs (14). The Fc-portion of IgG undergoes post-translational modification (15), which results in variable glycosylation patterns of IgG antibodies (16) and determines some of Fc-mediated effector functions (17). Manipulation of IgG by enzymes allows direct cleavage of specific carbohydrates from the Fc-portion (18). Hereby N-glycan at position Asn-279 is of major interest, as this glycan has been shown to mediate several effector functions of IgG (15, 19-21).

In this study we show data indicating that N-glycan Asn-279 has relevant impact on the effector function of platelet AAbs (obtained from well characterised ITP patients) by in vitro experiments and in vivo by taking advantage of a NOD/SCID mouse model (22-24), which allows assessment of the interaction of human antibodies with human platelets in vivo.

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Material and methods

Antibodies

Platelet-specific AAbs were isolated from sera of ITP patients (n=15) who were selected from a previously described cohort (25). Antibody detection was performed using a glycoprotein-specific assay (the monoclonal antibody–specific immobilisation of platelet antigens assay, MAIPA) (26, 27). In MAIPA, monoclonal antibodies (mabs) Ig5, SZ22, FMC25, Ig9, Ig18, D2 and B1G6 specific for platelet-membrane glycoproteins (GP) GPIib/IIa, GPIIIb, GPIb/IX, GPIa/IIa, CD31, CD109 and beta-2-microglobulin of HLA-class I antigen, respectively, were used as capture antibodies.

N-glycan modification

N-glycan was enzymatically removed from IgG by incubation with endoglycosidase F (EndoF; Native Protein Deglycosylation Kit, Sigma-Aldrich, Munich, Germany) (1/100 v/v) for 24 hours (h) at 37°C. Thereafter, IgGs were isolated by the Melon gel monoclonal IgG purification kit (Thermo Fisher Scientific).

To exclude cleavage of protein parts of the antibodies, a reversible staining was performed prior to the lectin blotting using Sepharose S staining as previously described (28). Briefly, a total of 1 µg IgG fraction was resolved in 4-12% precast gradient SDS-PAGE gels, transferred to nitrocellulose membranes (Invitrogen GmbH, Karlsruhe, Germany) under reducing conditions. Thereafter, proteins were transferred to nitrocellulose membranes and incubated with 0.1% Ponceau S solution (w/v) in 5% acetic acid for 15 minutes (min). Prior to lectin blotting, gels were destained in Tris-buffered saline (TBS) containing 0.05% Tween. N-glycan was detected by biotinylated lens culinaris agglutinin (LCA, 50 ng/ml, Sigma-Aldrich) for 45 min at room temperature (RT). Membranes were washed 10 times with TBS supplemented with 0.05% Tween. Subsequently, peroxidase-conjugated streptavidin (1 mg/ml, Sigma Aldrich, Munich) was added in a final concentration of 1.0 µg/ml for 30 min at RT. After washing, bound LCA was visualised by enhanced chemiluminescence (ECL) detection (GE Healthcare, Munich, Germany).

Isolation of monocytes

Monocytes were isolated from peripheral blood samples of healthy donors (blood group O) using AutoMACS™ technology (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. In brief, peripheral blood mononuclear cells (PBMCs) were purified from whole blood using Ficoll-Paque™PLUS (GE Healthcare Bio Sciences, Uppsala, Sweden). PBMCs were adjusted to 1x10^6 cells/µl in PEB (2.5 mM DPBS/EDTA, 0.5% BSA). Eighty µl PBMC were then incubated with 20 µl of anti-CD14-microbeads (Miltenyi Biotec) for 15 min at 4°C. After a wash step (2 ml PEB, 10 min 4°C at 55 g), monocytes were purified using AutoMACS™. For the platelet phagocytosis assay, isolated monocytes were adjusted to 1x10^5 cells/µl (in 10% FCS-RPMI, PAA Laboratories, Pasching, Austria).

Platelet isolation and labelling

Platelet-rich plasma (PRP) was obtained from 10 ml citrated whole blood of the monocyte donor as previously described (28) and adjusted to 1x10^5 cells/µl. Platelets were then labelled using an intracellular dye CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate; Invitrogen). One hundred µl of platelets (10x10^6 cells) were incubated with CMFDA (2 µl of 1 mM CMFDA in 0.2% DMSO) for 45 min at RT. After washing, platelets were adjusted to 1x10^5 cells/ml in PEB. Aliquots of 100 µl labelled platelets were then incubated with 50 µl IgG fraction from ITP patients (30 min, 37°C).

Antibody-mediated platelet phagocytosis assay

Oposioned, FITC-labelled platelets were incubated with 1x10^5 monocytes/µl at a ratio of 1: 5 for 2 h at 37°C under agitation. Thereafter, monocytes were labelled with 5 µl PE-conjugated anti-CD14 mab (Becton Dickinson, Franklin Lakes, NJ, USA). The adherent and non-internalised platelets were identified by adding 5 µl Alexa-Fluor 555 conjugated anti-human CD41 mab (Becton Dickinson) for 20 min at RT. Cells were washed once with PEB and resuspended in DPBS and analysed by flow cytometry (BD FACS Canto™, Becton Dickinson). The phagocytic activity was defined as percentage of FITC-positive, Alexa-Fluor negative monocytes out of all monocytes. Maximal phagocytic activity was defined as platelet phagocytosis induced by the GPIIb/IIa-complex specific mAb AP2 (2.5 µg/ml) and set as 100%. IgG fractions (native and Asn-279-deglycosylated) were tested in triplicate with platelets and monocytes from three different donors.

Confocal microscopy

Platelets were CMFDA-labelled and opsonised as described above, and incubated with isolated monocytes of the same donor. After incubation, monocytes were put in an ibiTreat microscopy chamber (Ibidi, Martinsried, Germany) and allowed to adhere for 30 min at 37°C. Adherent cells were washed twice with 200 µl PBS and fixed in 2% PFA (Sigma-Aldrich). After washings, anti-CD14 mAb (BioLegend, Fell, Germany) was added at 100 µl/well and incubated overnight at 4°C. After washings, Alexa Fluor 555-labelled anti-mouse antibody and Hoechst 33342 (Invitrogen) were added to stain the membrane and cell nucleus of the monocytes, respectively, using a confocal laser scanning microscope (LSM510-Meta, Carl Zeiss Microlmaging GmbH, Jena, Germany).
Complement consumption and fixation assay

To study the effect of AAb-associated N-glycan on complement activation, samples were tested in a complement fixation assay as previously described, with minor modifications (25). Briefly, IgG from heat-inactivated serum was incubated with platelets from healthy donors (5x10⁴/µl). Two µl of this suspension were incubated with 2 µl guinea pig complement (Dade Behring, Newark, DE, USA) for 1 h at 37°C. Thereafter, 2 µl of sensitised sheep erythrocytes (concentration 2x10⁹/µl, Dade Behring) were added for 30 min at 37°C. Complement-induced haemolysis of the indicator cells (test red cells) was evaluated microscopically and graded as follows: 4: more than 75% haemolysis (i.e. no complement fixation to platelets); 3: 50-75% haemolysis; 2: 25-50% haemolysis; 1: less than 25% haemolysis (i.e. strong complement fixation to platelets). Accordingly, grade 4-3 reactions were designated negative, and grade 2-1 reactions were designated positive.

The ability of anti-platelet AAbs to bind complement factor C1q was tested by flow cytometry. Heat-inactivated IgG fractions were incubated with washed platelets (5x10⁴/µl, 30 min, 37°C). Untreated serum was subsequently added for 30 min as a source of complement. Platelets were washed once (700 g for 10 min at RT in 500 µl PBS/BSA supplemented with PGE1 at 100 ng/ml) and incubated with 40 µl of 1: 80 diluted FITC-labelled polyclonal anti-human C1q antibody (Dako Deutschland GmbH, Hamburg, Germany) and assessed by flow cytometry. Serum from a healthy blood donor was used as negative control; serum containing complement-activating HLA-class I-antibodies was used as positive control.

Analysis of antibody-mediated destruction of human platelets in NOD/SCID mice

The capability of AAbs to eliminate human platelets was analysed using the NOD/SCID mouse model as previously described, with minor modifications (23, 29). Since sera of NOD/SCID mice have no complement activity (30), preparation of human platelets was modified to distinguish complement-dependent and -independent platelet clearance. PRP was collected and supplemented with 50 ng/ml PGE1 and spun at 700 g for 10 min. The pellet was resuspended with complement-free or normal serum (Sigma-Aldrich) supplemented with 50 ng/ml PGE1 and 3.8% sodium citrate, and the platelet concentration was adjusted to 2x10⁸/ml. Two hundred µl resting platelets were injected into the retro-orbital plexus of age- and sex-matched NOD/SCID mice (stock number 001303; The Jackson Laboratory, Bar Harbor, ME, USA). After 30 min, a blood sample was collected to establish a baseline of the human platelet count in the mouse circulation. Subsequently, mice were injected intraperitoneally with 200 µl of sterile DPBS containing native or deglycosylated IgG fractions. Aliquots of 20-50 µl blood samples were taken periodically (after 60, 180 and 300 min) via tail bleeding, and the kinetics of platelet clearance were analysed using

Table 1: Clinical manifestation and laboratory data from ITP patients included in this study. MAIPA results are indicated as optical density (OD); sera were considered to be reactive when OD was 0.2 or above. Complement fixation was evaluated as outlined in Material and methods.

<table>
<thead>
<tr>
<th>Case</th>
<th>Clinical manifestation</th>
<th>Plt count (range x 10⁹/l)</th>
<th>Bleeding symptoms</th>
<th>Direct MAIPA (bound antibodies, OD)</th>
<th>Indirect MAIPA (free antibodies, OD)</th>
<th>HLA class I</th>
<th>Complement fixation</th>
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<td>0.13</td>
<td>0.34</td>
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</tr>
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</table>
flow cytometry as described previously. Animal experiments were performed with approval of the local authorities.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee.

**Statistical analysis**

The statistical analysis was performed using Prism, Version 5.0 (GraphPad, La Jolla, CA, USA). Non-parametric tests were used when data failed to follow a normal distribution. All analyses were two-tailed, and a p-value of <0.05 was assumed to represent statistical significance.

**Results**

**Ex vivo modification of autoantibodies from ITP patients**

Antibody detection was performed using the glycoprotein-specific immune assay (MAIPA). Among the 15 ITP patients, five patients tested positive for AAbs against GPIIb/IIIa, five patients tested positive for AAbs against GPIb/IX, and five patient tested positive for both (▶Table 1). None of the sera contained anti-platelet IgM AAbs or antibodies against HLA-class I antigens. All controls tested negative for platelet-reactive auto- or alloantibodies by MAIPA and HLA antibody screen.

After glycan modification by EndoF, Ponceau S staining under reducing conditions showed the presence of two bands with molecular masses of 50 kDa and 25 kDa representing the heavy and light chains, respectively. LCA blotting showed a strong signal of the heavy chain of untreated IgG and complete removal of N-glycan by EndoF treatment. ▶Figure 1 shows a representative example of results found for all AAbs used in this study.

To exclude alterations of the paratope of AAbs as a result of EndoF treatment, IgG-binding to platelets was compared between treated and untreated antibodies using flow cytometry. Antibody binding to platelets was not affected after deglycosylation (mean fluorescence intensity (MFI): 191, range 90-212 vs 156, range 74-191, p=0.502), indicating that the removal of N-glycan from the Fc-fragment did not change the autoantibodies' binding specificity (▶Figure 1).

Autoantibody-mediated platelet phagocytosis depends on the N-glycan status

To investigate whether the removal of N-glycan does affect the autoantibodies’ capability to induce platelet phagocytosis, CMFDA-labelled platelet were opsonised with native or EndoF-treated AAbs and incubated with monocytes. Signals from adherent, but not internalised platelets were excluded by a second platelet labelling with Alexa-Fluor 555-anti-CD41. To allow comparability between different experiments performed using different donor cells, phagocytic activity was expressed as percentage of phagocytosis induced by the GPIIb/IIIa-complex specific mAb AP2 (phagocytic activity 100%). Native AAbs induced a median phagocytic activity of monocytes of 8% (range 6-13%, n=15), which was significantly higher than that induced by deglycosylated AAbs (0.8%, range 0.1%- 1.1%, p<0.001). In contrast, the phagocytic activity of monocytes induced by deglycosylated AAbs did not differ from the one induced by native IgG of healthy donors (median 0.8%, range 0.0- 1.2%; n=6, p=0.79) or native IgG of non-ITP thrombocytopenic control patients (median 1.0%, range 0.7%- 1.2%; n=4; p=0.13) (▶Figure 2 A).

Confocal microscopy confirmed the results obtained by the phagocytosis assay. As shown in ▶Figure 2 B, CMFDA-platelet (green) and monocytes (red) are located apart from each other after incubation with isotype mab. In contrast, platelets (green) opsonised with AAbs are located between cell membrane (red) and cell nucleus (blue) (data are representative for n=3 independent experiments). Our results indicate that opsonised platelets are completely ingested into the cytoplasm of the monocytes (▶Figure 2 B).

The removal of N-glycan from the Fc-part of AAbs resulted in a significant reduction of the antibodies’ capability to induce platelet phagocytosis as determined in both flow cytometry (median phagocytic activity after deglycosylation: 0.6%, range 0.1%- 0.9%, p=0.003) and confirmed by confocal microscopy (▶Figure 2 A and B).
Deglycosylated autoantibodies are not capable of complement activation in vitro

In the complement fixation assay, complement consumption was induced by the IgG fractions of the sera from seven ITP patients. In contrast, none of the control IgG fractions induced complement activation. Complement consumption was completely lost after deglycosylation of AAbs (Table 2). In flow cytometry, complement-activating platelet AAbs fixed C1q to platelets, as detected by anti-C1q secondary antibody (MFI: 16.4, range 11.3-19); C1q binding was inhibited after deglycosylation (MFI: 4.9, range 3.7-5.7, p=0.017). IgG fractions from non-ITP thrombocytopenic control patients (n=4) and healthy donors (n=4) did not induce C1q binding (MFI: 5, range: 4.2-5.8). These results indicate that C1q binding to platelets and AAb-mediated complement consumption depends on the presence of N-glycan on the autoantibodies.

IgG N-glycan modulates in vivo platelet destruction

After injection of human platelets into NOD/SCID mice, approximately 100x10⁶/µl human platelets (~15% of total circulating pla-
N-glycan affects platelet destruction in ITP

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Complement

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Table 2: Complement consumption and fixation data. Complement consumption and fixation was determined as outlined in Materials and methods. In the complement fixation assay with haemolysis of indicator erythrocytes as a read-out, haemolysis is graded from 1 to 4. Accordingly, "1" indicates strong complement fixation to platelets, and "4" indicates no complement fixation to platelets. For C1q binding to platelets, mean fluorescence intensity (MFI) values are given. All data are mean values from n = 3 independent experiments. Note that the ability to fix complement was lost after deglycosylation of sera no. 1, 2, 5, 7, 9, 10 and 12. Each control group included n = 4 different individuals. –, not tested.

<table>
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<th>Sample</th>
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<th>C1q binding assay</th>
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</thead>
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<td>deAAb</td>
</tr>
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Discussion

This study indicates that: 1) N-glycan is required for platelet autoantibody-induced phagocytosis of platelets by monocytes; 2) N-glycan of platelet AAbs is required to fix C1q and to trigger complement consumption; 3) both N-glycan dependent processes decrease the survival of human platelets in the presence of platelet AAbs in vivo.

Our study confirms a contribution of Fc-receptor mediated phagocytosis in antibody-mediated platelet elimination in patients with ITP (6, 8). We identified Asn-279 N-glycan as a relevant structure of anti-platelet AAbs in ITP, as its removal from autoantibodies blocks platelet phagocytosis by monocytes in vitro and strongly reduces platelet elimination in vivo. This is in line with previous studies on the role of N-glycan moieties in IgG-Fc-receptor interactions (18).

In contrast to the role of IgG N-glycans for phagocytosis of opsonised platelets, the role of IgG N-glycan moieties and activation of the complement system in ITP is more controversial. Some investigators suggest that Asn-279 N-glycan might be relevant for initiation of the classical, but not the alternative, pathway of complement activation (31), and in ITP patients, complement activation has been associated with a low-responder status after splenectomy (25, 32). Other studies, however, question a major contribution of complement to the pathogenesis of ITP (33). Our study confirms this heterogeneity of AAbs in ITP patients. Some sera activated the complement system, while others had no effect. Interestingly, in complement activating AAbs, removal of the N-glycan abolished complement activation as well as C1q deposition on platelets. This strongly indicates that a subset of ITP AAbs induces the classical pathway of complement activation, which depends on Asn-279 glycan. However, this has been only shown in vitro under experimental conditions and little is known on the contribution of other pathways, i.e. Fc-receptor mediated phagocytosis and/or complement activation in vivo.

Therefore we employed an in vivo mouse model to verify the relative contribution of both pathways to platelet AAb-mediated thrombocytopenia. This showed that the mechanisms of platelet elimination by AAbs differ depending on the biological features of the AAbs. AAbs which fix complement predominantly induced platelet elimination by a complement-dependent pathway, since...
addition of complement accelerated the elimination of autoantibody-coated platelets from the circulation (Figure 3, upper row). In contrast, AAbs which do not fix complement primarily induce platelet elimination via Fc-receptor dependent phagocytosis. These findings may be one reason for the different response of ITP patients to different treatments. It is tempting to speculate that ITP patients whose AAbs do not activate complement may respond to splenectomy while those whose AAbs strongly activate complement might not. Confirming this hypothesis, however, requires well designed clinical studies.

Recently, we and others showed that in a significant number of patients with chronic ITP, platelet autoantibodies are capable of activating the classical complement pathway even if autoantibodies were undetectable by conventional methods (25, 32). We speculate that the ability of ITP antibodies to activate complement might, at least partially, have impact on the clinical course (34). This aspect is of special interest, since taking the newly developed anti-complement agents into account, it is presumable that new therapeutic approaches of ITP should target complement factors especially in the acute phase. Eculizumab, for example, was shown to reverse thrombocytopenia in patients with catastrophic antiphospholipid syndrome by inhibiting the terminal components of complement (35).

As our study shows that AAbs in ITP patients seem to be heterogeneous in their effector functions, clinical studies in ITP should aim to assess these characteristics. This could probably lead to the identification of subgroups of ITP patients with increased response(s) to a specific treatment approach. Our study provides some tools for better characterising ITP AAbs in vitro as well as in vivo.

In conclusion, we have demonstrated that the removal of N-glycan from autoantibodies interferes with Fc-mediated phagocytosis and complement activation and thereby prolongs platelet survival in vivo. Our results shed light on the heterogeneity of platelet autoantibodies and the potential relevance of complement in ITP and may open the way towards new therapeutic options.

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Figure 3: N-glycan is required for in vivo platelet destruction. The impact of N-glycan on platelet clearance in vivo was analysed using the NOD/SCID mouse model. Since sera of NOD/SCID mice have no complement activity, preparation of human platelets was modified to distinguish complement-dependent and -independent platelet clearance. Human platelets were injected in presence (black lines) or in absence (dashed lines) of exogenous human complement. Thereafter, native IgG fractions from control sera (n=4) or from ITP sera identified in vitro as either non complement-activating (n=8) or as complement-activating (n=7) were injected (upper row). Platelet survival was investigated after 1, 2 and 5 h. In a second set of experiments, deglycosylated IgG fractions obtained from the same control and ITP sera were injected under identical conditions (lower row). Note that AAbs with the ability to activate complement in vitro induced more pronounced platelet clearance in the presence of complement compared to the clearance in the absence of complement (upper row, third column) and that in vivo AAb-induced platelet destruction was N-glycan dependent in this model.
What is known about this topic?
- Immune thrombocytopenia (ITP) is caused by increased platelet destruction.
- Autoantibodies in ITP can induce platelet destruction by several mechanisms.
- Some of the autoantibodies mediate phagocytosis and others activate complement.

What does this paper add?
- N-glycan of platelet autoantibodies is required for platelet autoantibody-induced phagocytosis.
- N-glycan of platelet autoantibodies is required to fix C1q and to trigger complement consumption.
- Both N-glycan dependent processes decrease the survival of human platelets in the presence of autoantibodies in vivo.

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Conflicts of interest
None declared.

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