Detection of anti-platelet antibodies in immune thrombocytopenia by flow cytometry

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Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disorder characterised by low platelet count (≤100x10^9/L), due to accelerated platelet destruction and/or impaired platelet production (Cines*, et al* 2009). Anti-platelet antibodies (APAbs) are detectable in ~60% of ITP patients, with glycoprotein Ib-IX and $\alpha_{IIb}\beta_3$ representing the major antigenic targets (Cooper 2017). APAbs can promoting platelet clearance and also diminish platelet formation (Lev*, et al* 2014). As well as APAbs, dysfunctional cellular immunity (e.g. abnormal T-cell function) can also contribute to the pathogenesis of ITP (Lambert and Gernsheimer 2017). Although recent advances in our understanding of ITP have led to the publication of guidelines (Neunert*, et al* 2011), ITP diagnosis and treatment remain challenging due to the patient variability and the fact that symptoms often correlate poorly with platelet count (or APAb titre) (Frelinger*, et al* 2015). Drugs such as dexamethasone, rituximab or thrombopoietin receptor agonists have improved remission rates (up to 60%). (Lambert and Gernsheimer 2017). The complex pathology and lack of diagnostic tests for ITP make treatment a challenge. The currently available APAb assays are limited to a few antigens, in the absence of the platelet membrane, which can cause loss of antigenicity and result in reduced sensitivity and specificity. These limitations, together with the complexity of assays can make them ineffective in the diagnosis and monitoring of ITP patients. Here, we describe a flow cytometry assay where APAbs are efficiently and reproducibly detected in patients with ITP.

We analysed samples from 66 patients with newly diagnosed, persistent or chronic ITP for APAbs. Rather than detecting antibodies against specific platelet antigens (e.g. $\alpha_{IIb}\beta_3$ or GPIbα) in ELISA-based assays or derivatives of these, we used flow cytometry to detect IgG bound to platelet surfaces. We first incubated washed healthy volunteer platelets with either ITP patient serum (Fig 1Ai) or total IgG isolated form ITP patient sera (Fig 1Aii), and thereafter detected bound IgG using FITC-conjugated anti-human IgG. However, we found little or no evidence for APAb on platelet surfaces in any of the samples analysed, even when an excess of serum or isolated IgG were used. We therefore hypothesised that there is little free APAb in the sera of ITP patients as they are sequestered on the patient platelets. ITP patients are variably thrombocytopenic. Despite this, APAbs could be readily detected on the majority of the ITP patient platelets by direct labelling (Fig 1Aiii), even in patients with platelet counts as low as 5x10^9/L. A major drawback with this approach is that each patient platelet sample must be prepared and analysed following collection. To enable collection/storage of samples, we prepared ITP patient platelets, and then eluted APAbs from the platelet surfaces using glycine. APAbs from platelets were detected by incubating these samples with healthy washed platelets (Fig 1Aiv), and with FITC-conjugated anti-human IgG, as before. Data using platelet-eluted antibodies were indistinguishable from those in which APAbs were detected directly on ITP patient platelets (Fig 1Aiii & iv). Using platelet-eluted samples, 69.7% out of the 66 patients tested positive at least once for APAbs. This number increased (72.5%) when we only include patients for whom we have multiple longitudinal samples (n=40) (Fig 1B). ITP patients exhibited varying degrees of APAb positivity with (mean fluorescence intensities) MFI of 8.0 ±1.9 for weakly positive patients (group 2) to 76.5 ±29.8 for very strongly positive patients (group 4) (Fig

1B & Table 1). No APAbs were detected in 27.5% of the patients (group 1) (MFI of 4.6 ±0.8; Figure 1B), or in samples from healthy controls (n=54) (MFI of 4.0 ±0.8), highlighting the 100% specificity of the test, which is a major improvement when comparing previous studies/assays (Liu*, et al* 2011, Najaoui*, et al* 2012, Romero-Guzman*, et al* 2000). The percentage of patients that tested negative with our assay is in line with numbers reported using other flow cytometry or MAIPA (Cines*, et al* 2009). However, as APAb positivity often fluctuated appreciably over time, we may overestimate the number of negative patients for APAbs. 20/66 patients were APAb negative; of these, 9 patients had just a single sample for analysis. It is likely, therefore, that APAbs would be detected in some of these patients through analysis of longitudinal samples.

Analysis of longitudinal ITP patient samples revealed some interesting case reports (Figure S1). Patient A, responded to TPO agonist treatment, which was accompanied by a progressive reduction in APAb positivity suggesting their involvement in thrombocytopenia (Figure S1). However, a rapid decline in platelet count at week 28 in the absence of detectable APAbs suggests that additional disease mechanisms are also important in the pathogenesis of ITP in this patient. More clearly for patient B, we found that this patient always tested positive for APAbs, but that platelet count did not inversely correlate with APAbs. This further challenges the link between APAb and the pathogenesis of ITP. Consistent with this, we found that there was the same proportion of patients with low (<150x10^9/L) or normal platelet counts (>150x10^9/L**)** in each ITP patient category tested (Table 1). This underscores the complexity in diagnosing patients with ITP based on APAb and platelet count. This is also true for treatment, as there is a poor correlation between APAbs levels and prediction of rituximab responders (Arnold*, et al* 2017). It remains unclear, therefore, whether APAbs are a cause or a consequence of the disease, and what the influence of APAbs on platelet clearance and production is.

Although our assay requires the time consuming preparation of washed platelets, it allows storage of APAb and more importantly offers a sensitive and reproducible method to directly or indirectly detect APAb in ITP patients. APAbs were detected in ~70% of the ITP samples analysed, but this may still represent an underestimation of the proportion of APAb positive patients. Longitudinal analysis of APAb positivity/negativity will assist in identifying those patients whose APAbs are pathogenic, and those whose disease is manifest through alternative pathogenic mechanisms. Further studies are now warranted to correlate APAb with different treatment regimens and their detection upon the likelihood of clinical relapse.

Authorship

Contribution: A.T-O and I.I.S-C designed and performed experiments, analysed data and wrote the paper; NC designed the research and reviewed the manuscript; J.T.B.C. designed the research, and wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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Table 1. Proportion of ITP patients with low or normal platelet counts.

Group	APAb[a]	MFI[b]	Platelet count[c]		no. Samples[d]
			>150x10^9/L	<150x10^9/L	
1	-	4.6±0.8	28 (27.4%)	74 (72.5%)	102
2	+	8.0±1.9*	22 (38.6%)	35 (61.4%)	57
3	++	22.8±7.9****	13 (21%)	49 (79%)	62
4	+++	76.54±29.8****	8 (32%)	17 (68%)	25
Control	-	4.0±0.8	54 (100%)	0	54

[a]APAb (Anti-platelet antibodies)

[b]MFI (Mean Fluorescence Intensities of human anti-IgG-FITC; mean ± SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-test using Control group as reference. * $p<0.05$; **** $p<0.0005$

[c]Platelet count: number of samples processed are indicated with percentage in brackets exhibiting normal or low platelet count. Statistical analysis was performed using Chi square test; $p>0.05$.

[d]no. Samples: Represent the total number of samples with platelet count available.

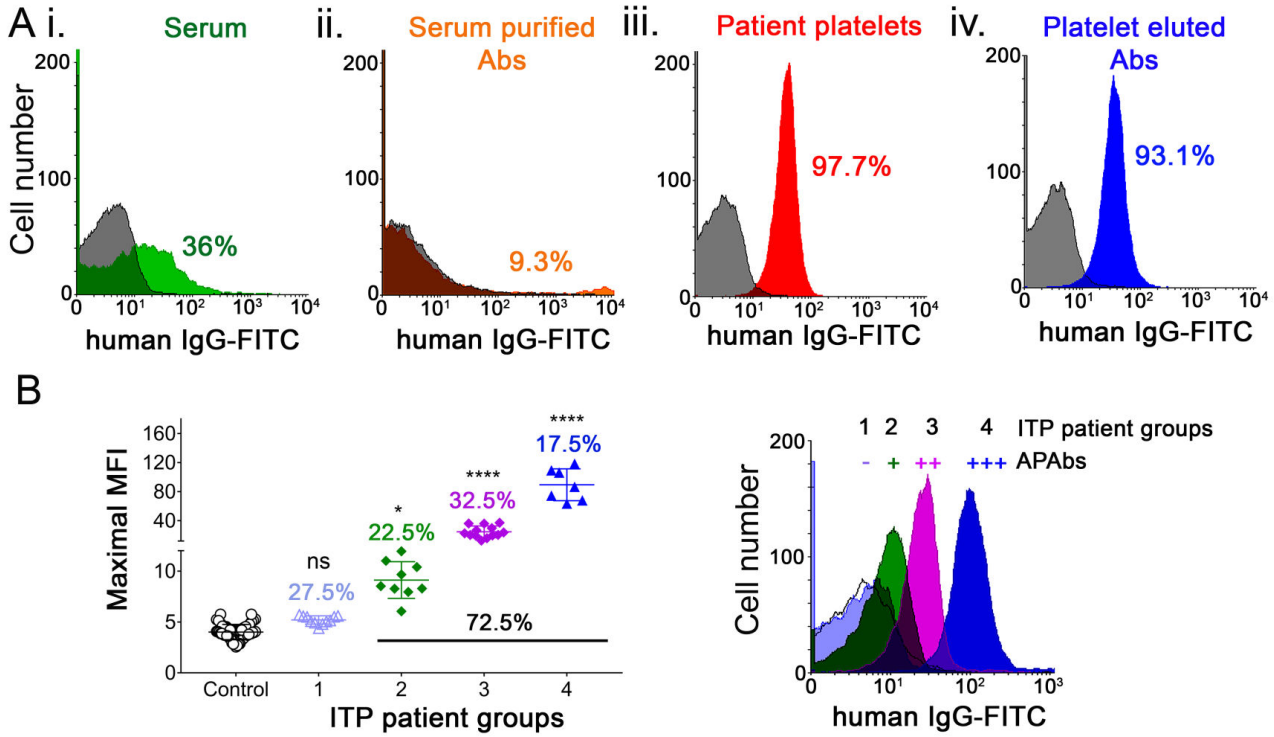


Figure 1. Determination of APAb in 66 patients with ITP by flow cytometry assay. APAbs were detected with FITC conjugated goat anti-human IgG Ab directly on patient platelets (Aiii) or indirectly on control platelets pre-incubated with serum, serum purified antibodies or platelet purified Abs (Ai-ii & iv). (Ai-iv) Representative flow cytometry analysis of different samples collected on the same day from the same patient (coloured area) or healthy donor (grey area). The percentage of the platelets with APAbs is indicated for each histogram. (B) Graph representing the maximal Mean Fluorescence Intensities (MFI) (mean ± SD) for all patients with multiple samples and grouped into 4 different categories (left). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-test using Control group as reference. * $p<0.05$; **** $p<0.0005$. Representative flow cytometry analysis of four patients with ITP from each category (right).

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Supplementary materials and methods

Blood collection and platelet preparation

Ethics for the following study that included 66 patients (aged 19-84) with ITP attending immune haematology clinic at the Hammersmith Hospital was granted by the Imperial Ethics committee. Patient blood was collected in 3.2% sodium citrate (for platelet/plasma analyses), or in serum tubes for serum IgG analyses. Platelet counts were obtained using an automated cell counter (Sysmex XE2100) or by flow cytometry using calibrated beads (Saxon Europe). Washed platelets were prepared within 2 hours of blood collection (Cazenave*, et al* 2004) and used on the day of collection. Briefly, platelet-rich plasma was obtained by centrifugation at 150x*g* for 10 min at room temperature (RT). Platelets were resuspended in modified tyrode's buffer supplemented with 0.35% bovine serum albumin, 100nM Prostaglandin E1 and 75 mU/ml Apyrase and centrifuged at 1500x*g* for 10 min at RT. Platelets were washed twice as above and resuspended in modified tyrode's buffer for further analysis.

Platelet autoantibody testing

Total immunoglobulins (Igs) from ITP patient sera was obtained using Thiophilic Adsorption resin (ThermoFisher). APAbs were also obtained from ITP patient platelets by eluting bound Igs from washed platelets using 0.1M glycine, pH 2.5 and an equal volume of 0.5M Tris, pH 8.0. Total Igs isolated from normal sera, and eluted from healthy volunteer platelets were used as controls. Flow cytometry was used to identify APAbs using a direct labelling method consisting of incubating patient platelets with a FITC-conjugated goat anti-human IgG Ab (Fc specific; Invitrogen) or using an indirect method using patient sera, serum-purified Ig or platelet-eluted Ig incubated with healthy donor platelets from O-blood group. Briefly, healthy platelets (10^6/sample) were incubated at 37°C for 1 hour either with healthy or patients' sera, serum-purified Ig or platelet-eluted Ig (samples were titrated to ensure maximal sensitivity), washed and bound APAbs detected with FITC-conjugated goat anti-human IgG Ab. Platelets were gated on their forward and side scatters and 10,000 events were acquired using a FACSCalibur flow cytometer (BD Biosciences). The fluorescence threshold to determine the positivity of a sample (presence of APAbs) was defined by the average of mean fluorescence intensity (MFI) of platelets obtained with control samples ±2 SD.

Statistical analysis

For APAb detection, results are presented as mean MFI ±SD for each patient category and healthy controls, and statistical analysis performed using one-way ANOVA followed by Dunnett's post-test. Correlation between data obtained from patient platelets and from platelet-eluted Igs were calculated by the Spearman rank correlation test. All statistical analyses were performed using GraphPad Prism 7.0.

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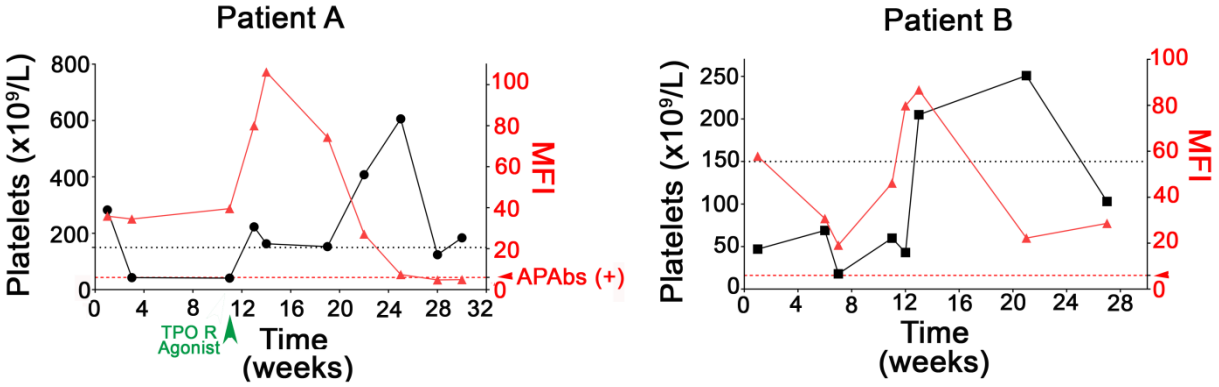


Figure S1. Examples of longitudinal studies for platelet counts and APAbs detection in two patients with ITP. APAbs were detected with FITC conjugated goat anti-human IgG Ab indirectly on control platelets pre-incubated with platelet eluted Abs. The MFI threshold of 5.7 determining the presence of APAbs is represented by a red dotted line. A normal platelet count of 150x10^9/L is represented by a black dotted line.