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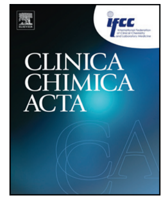

An improved flow cytometric immunobead array to detect autoantibodies in plasma from patients with immune thrombocytopenic purpura

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ABSTRACT

Background: Autoantibodies against platelet glycoproteins (GPs) play an important role in immune thrombocytopenic purpura (ITP). This study was to develop an improved flow cytometric immunobead array (FCIA) assay to detect platelet autoantibodies in ITP patient plasma.
Methods: Plasma samples were isolated from 71 ITP patients and 136 non-ITP controls and incubated with platelets from normal individuals. After washing, platelets were lysed and the platelet lysates were incubated with polystyrene microbeads coupled with monoclonal antibodies against human GPs IX (SZ1), Ib (SZ2), IIIa (SZ21), IIb (SZ22), and P-selectin (SZ51). Platelet GP–autoantibody complexes were detected by flow cytometry using a FITC-labeled secondary antibody.
Result: Autoantibodies against platelet GPIb, GPIIb, GPIIIa, GPIX and P-selectin were detected in plasma from ITP patients, as indicated by high mean fluorescent intensity values when microbeads with antibodies SZ1, SZ2, SZ21, SZ22, and SZ51 were used. In ROC analysis, values of the area under the curve were 0.74, 0.83, 0.80, 0.79 and 0.87, respectively. Compared with the previously reported assays, this new FCIA eliminated the need of isolating platelets from ITP patients without compromising assay sensitivity and accuracy in predicting ITP.
Conclusion: This simplified FICA assay may be more suitable for ITP diagnosis in clinical laboratory settings.
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1. Introduction

Immune thrombocytopenic purpura (ITP) is an acquired hematological disorder characterized by isolated thrombocytopenia (platelet counts $<100 \times 10^9$/l) without apparent causes account for the reduced platelet counts [1,2]. To date, the mechanism underlying ITP is not well understood. A number of factors may contribute to the disease by increasing platelet destruction and/or reducing platelet production [3–11]. Studies show that autoantibodies against platelet glycoproteins (GPs) are one of the most important contributing factors [4,5,12]. In patients with ITP, commonly detected autoantibodies include those against GPIb-IX and GPIIb-IIIa complexes, which are abundant GPs on the platelet surface and important for platelet adhesion and aggregation [5,13]. When the autoantibodies form complexes with the platelet GPs on the cell membrane, platelets are more likely targeted for endocytosis by macrophages and lysis by complements [12,14]. The autoantibodies against the platelet GPs and autoreactive T cells may also inhibit megakaryocyte differentiation in the bone marrow [15,16]. Thus, the autoantibodies against platelet GPs may contribute to ITP by reducing platelet generation in the bone marrow and lowering platelet counts in peripheral blood. Low platelet counts impair hemostasis, thereby causing bleeding in patients.

The diagnosis of ITP is based on medical history, physical examination, blood chemistry, and peripheral blood smear to exclude other possible causes of thrombocytopenia [2,6,17]. Assays such as ELISA-based monoclonal antibody immobilization of platelet antigen (MAIPA) assay and immunobead-based radioimmune assay (RIA) have been developed to detect platelet autoantibodies [18,19]. These assays provide useful information about autoantibodies against specific platelet antigens. Technically, however, these assays are time-consuming, which limits their use in clinical laboratory settings.

Recently, flow cytometric immunobead array (FCIA) has been developed for ITP diagnosis. This technique uses flow cytometry and antibody-coated polystyrene microbeads to detect autoantibodies against multiple platelet antigens in single tubes [20–22]. Compared with the traditional ELISA and RIA methods, FCIA-based assays require smaller sample volumes and have quicker and simpler experimental

Abbreviations: FCIA, flow cytometric immuobead array; FITC, fluorescein isothiocyanate; GAH, goat anti-human; GAM, goat anti-mouse; GP, glycoprotein; ITP, immune thrombocytopenic purpure; MAIPA, monoclonal antibody immobilization of platelet antigen assay; MFI, mean fluorescent intensity.

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procedures. In a recent study, we showed that an FCIA assay using multiple antibodies against platelet GPs had better sensitivity and accuracy than MAIPA in detecting autoantibodies in ITP patients [20].

The FCIA assays used in our previous studies and reported by others require isolation of platelets from ITP patients [20–22], which could be difficult especially in patients with low platelet counts. In this study, we report a modified FCIA assay that uses plasma, but not platelets, from ITP patients for the detection of autoantibodies. By avoiding platelet isolation, this new FCIA assay may be more suitable for clinical laboratories in the diagnosis of ITP.

2. Materials and methods

2.1. Antibodies and polystyrene microbeads

Monoclonal antibodies SZ1, SZ2, SZ21, SZ22 and SZ51 against human platelet GPIX, GPIb, GPIIIa, GPIIb, and P-selectin, respectively, were generated and characterized, as described previously [23–27]. Labeled secondary antibodies, including fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG (FITC-GAH) and anti-mouse IgG (FITC-GAM) polyclonal antibodies, were purchased from Bechman-Coulter (Suzhou, China). Polystyrene microbeads (4 μm in diameter) labeled with dyes of different fluorescent intensities were purchased from Spherotech (Lake Forest, IL).

2.2. Antibody-coupled microbeads

The methods coupling microbeads with antibodies were described previously [20]. Briefly, affinity purified monoclonal antibodies SZ1, SZ2, SZ21, SZ22 and SZ51 (160 μg each) were prepared in a carbonate buffer (pH 9.5) and incubated with 1×10^6 microbeads labeled with dyes of different fluorescent intensities on a rotator at 4 °C. After 12–14 h, the microbeads were washed three times with PBS in the presence of 0.05% Tween-20. The antibody-coupled microbeads were stored in PBS containing 0.02% sodium azide at 4 °C. The stored microbeads were tested each month for up to 10 months for their stability.

2.3. Patient samples

This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University in Suzhou, China. All participants provided informed consent. Venous blood was collected from ITP patients ($n = 71$), who visited the hospital between June and December 2012. The patients included 30 males and 41 females, aged from 5 to 68 y (average 36 y). All the patients had platelet counts below 100 $\times 10^9$/l. The American Society of Hematology guideline was used for the diagnosis of ITP [1]. Blood samples from non-ITP patients ($n = 71$), whose diagnosis included myelodysplastic syndrome ($n = 17$), anemia ($n = 23$), leukemia ($n = 19$), and paroxysmal nocturnal hemoglobinuria ($n = 12$), were used as controls. Additional control blood samples were obtained from normal subjects ($n = 65$; 40 males and 25 females), aged from 21 to 58 y (average 36 y) who underwent health check-ups at the hospital. Platelet counts in these non-ITP and normal individuals were $>100 \times 10^9$/l.

2.4. Plasma preparation

Venous blood (2 ml) was collected in tubes containing spray-coated EDTA as an anticoagulant (BD Diagnostics) and centrifuged at 3000 *g* at room temperature for 5 min, as described previously [28]. After the centrifugation, platelet-poor plasma was collected and transferred into new tubes. Aliquots of plasma samples were used in FCIA and MAIPA assays described below.

2.5. FCIA assays

A modified FCIA assay was developed, which used plasma instead of platelets from ITP patients. In this assay, referred as indirect FCIA assay hereafter, aliquots of platelet-poor plasma (110 μl) from ITP patients were mixed with normal platelets (1×10^9/ml in 110 μl) from healthy volunteers at room temperature for 1 h. Platelets were washed three times with phosphate-buffer saline (PBS) containing 0.05% EDTA and lysed in a PBS buffer (110 μl) containing 1% Triton X-100. Samples were centrifuged at 3000 ×g for 20 min and soluble platelet lysate

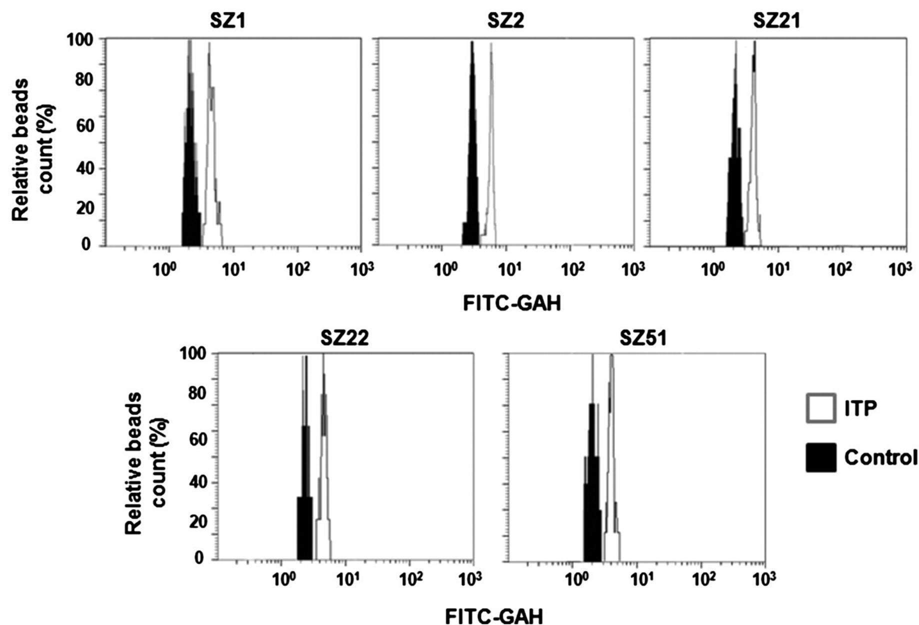

Fig. 1. Detection of platelet autoantibodies in ITP patient plasma. Plasma from ITP patients (*open peaks*) or normal controls (*black peaks*) were incubated with normal platelets from healthy volunteers. After washing, platelets were lysed and incubated with microbeads coupled with monoclonal antibodies SZ1, SZ2, SZ21, SZ22 or SZ51. Autoantibodies against platelet GPs were detected by flow cytometry using an FITC-labeled secondary antibody.

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