Special Communication

Flow Cytometry Method for Platelet-Specific Antibody Detection by Acid Treatment through Human Leukocyte Antigen-1 Removal

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Abstract

Background: Platelet (PLT) transfusion has been a crucial for clinical blood transfusion and bleeding prophylaxis. Identification of antibody against PLT-specific antigen is important for accurately diagnosing posttransfusion purpura, neonatal alloimmune thrombocytopenia, and platelet refractoriness due to PLT-specific antibodies. However, the presence of anti-human leukocyte antigen (HLA) antibody makes the detection of PLT-specific antibody more complex. **Materials and Methods:** The influence of treatment of PLTs with phosphate-buffered solution (PBS) or citric acid at pH 3.0 on the expression of human-specific antigen and HLA Class I was tested by flow cytometry. PLTs were treated with citric acid at room temperature for 5 min, and all solutions were precooled at 4°C. **Results:** Acid treatment reduced more than 90% of HLA Class I complexes when compared with PBS-treated ones on the surface of PLT membrane without any significant cell damage. The antigenicity of HLA Class I was lowered with time, but the antigenicity residue was little with PLT fragments after more than 5 min. However, human-specific antigen involving CD61 was not decreased after appropriate acid treatment, which was confirmed by corresponding monoclonal antibody. The use of standard serum from National Institute of Biological Standards and Control (NIBSC) containing only anti-human PLT antigen-1a antibody or anti-HLA antibody-positive control was applied to confirm the effect of acid treatment on PLT antigen. **Conclusion:** These findings suggested that acid treatment method could be useful for detecting PLT-specific antibodies, guiding clinical transfusion.

Keywords: *Acid treatment, flow cytometry, human leukocyte antigen-1, platelet transfusion, platelet-specific antibodies*

Received: 28-Sep-2019.
Revised: 28-Sep-2019.
Accepted: 28-Sep-2019.
Published: 17-Oct-2019.

Introduction

Platelet (PLT) transfusion has become an important approach for clinical therapy. Human PLTs are the smallest cellular members in human blood and act as a hemostatic agent for staunching bleeding through activation of coagulation cascade. The abnormal PLT count indicates bleeding risk, in which the normal level ranges from 100 × 10^9/L to 300 × 10^9/L. If normal level was below, then the condition is called as thrombocytopenia. The reasons for the decreased PLT count included both immune and nonimmune factors. Patients who underwent mismatched transfusion of blood possibly induce alloimmune response and produce antibodies against specific antigens, causing a failed transfusion.[1] The reasons for the induction of alloimmune antibodies were human leukocyte antigen (HLA) and human PLT glycoprotein antigens (HPA).[2] Therefore, the detection of PLT antibodies is of significant importance.

There are various methods to detect PLT antibodies, and each has their own merits and demerits. Monoclonal antibody-specific immobilization of PLT antigen (MAIPA) is recommended as a gold standard by the International Platelet Immunology Workshop

Access this article online

Quick Response Code:

Website: www.gjtmonline.com

DOI: 10.4103/GJTM.GJTM_61_19

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How to cite this article: Ma QQ, Zhu HJ, Rui-Shu L, Min F, Ping L. Flow cytometry method for platelet-specific antibody detection by acid treatment through human leukocyte antigen-1 removal. Glob J Transfus Med 2019;4:243-6.

for detecting antibodies, but the operation of MAIPA involves a cumbersome process and demands expertise. Furthermore, a commercial kit of MASPAT, Capture-P, is expensive and inconvenient for use in hospitals. The results are interpreted by aggregation of red blood cells, which is more subjective.

Anti-HPA antibodies often coexist with anti-HLA antibodies in a patient's serum, which is probably due to more immunogenicity of HLA than HPA. Studies have shown that it would interfere in detecting the HPA antibodies when HLA antibodies were present. Addition of citric acid is an effective way to remove HLA-1 on PLTs without causing death of the PLTs. Flow cytometry (FCM) is widely used in the medical field due to its sensitivity and convenience. Hence, FCM was used to confirm presence of specific HPA antibodies.

Materials and Methods

Platelet concentrates

The PLTs used in the experiment were obtained from healthy blood donors.

Acid treatment of platelets

Pooled PLTs were centrifuged and resuspended at a density of 1×10^5/μl in ice-cold citric acid buffer. The acid solution at pH 3.0 contained an equal volume of 0.263 M citric acid and 0.123 M Na_2HPO_4 with 1% bovine serum albumin. The treatment was completed by removing excess volume of ice-cold acid-citrate-dextrose buffer and washing thrice. The PLTs were then pelleted and recovered to original density. In the control group, the untreated PLTs were suspended in phosphate-buffered solution (PBS) by adjusting to the same confirm presence of specific HPA antibodies.[3]

Flow cytometry

The antibodies used were anti-CD62P BV421 anti-CD41 FITC anti-CD61 PE anti-HLA-ABC anti-IgG1 APC BD Biosciences, USA; anti-GP2b/3a, anti-GP1b Suda Saier immunizationbiotechnology Co Ltd, Jiangsu, China; and the standard serum with anti-HPA-1a (100 IU, 03/152) and/or anti-HLA antibodies (07/214) were obtained from NIBSC. FCM was performed by using a cell analyzer (BD FACSVerse, BD Biosciences, USA). FlowJo software (FlowJo Software, BD Biosciences, USA) was used to analyze the data.

Results

There is a tailing phenomenon observed in the acid-treated group when compared to the untreated group [Figure 1a], which suggested an increasing size or change of shape of the PLTs. The viability of PLTs was measured by FCM after citric acid treatment for 5 min at room temperature (RT). The results showed no statistically significant difference between the treated group and the untreated group for CD61 on the PLTs [Figure 1b]. Immunofluorescence test was applied to study whether citric acid treatment eliminated HLA, and the results showed that more than 95% of HLA-I was removed from the PLT surface by exposure to acid solution.

Next, the effect of acid treatment on other antigens/glycoproteins on PLTs was investigated. The expression level of GP2b/3a and GP3a showed no significant changes [Figure 2], indicating that low pH was more sensitive to HLA-I complex. To test if acid treatment induced the activation of PLTs, the expression of CD62P [Figure 1c] was measured, and the results varied from 10.2% ± 2.5% to 48.8% ± 5.4% following acid treatment. The activation was caused by exposure to acid solution, rather than by mechanical force during centrifugation or resuspending when compared to the control group.

To identify the method for detecting HPA antibodies, different gradients of standard serum-containing anti-HPA-1a antibody were allowed to react with PLTs by exposing them at pH 3.0. In the meantime, the AB

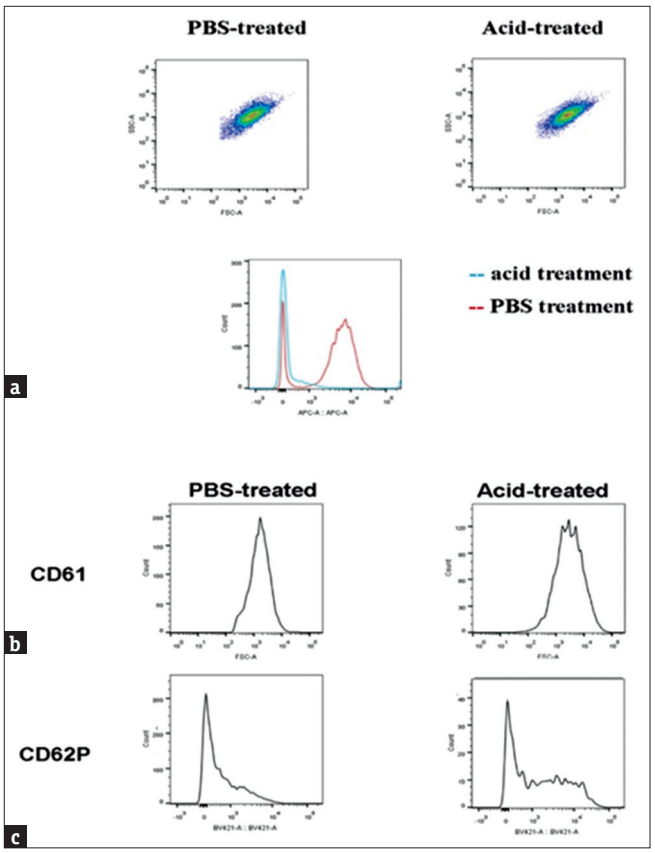

Figure 1: The viability of platelets by acid treatment. (a) Representative of forward scatter/side scatter plots of untreated and acid-treated platelets. (b) The residual of human leukocyte antigen-I by acid treatment. (c) CD61 and CD62P expression of platelet before and after citric acid treatment

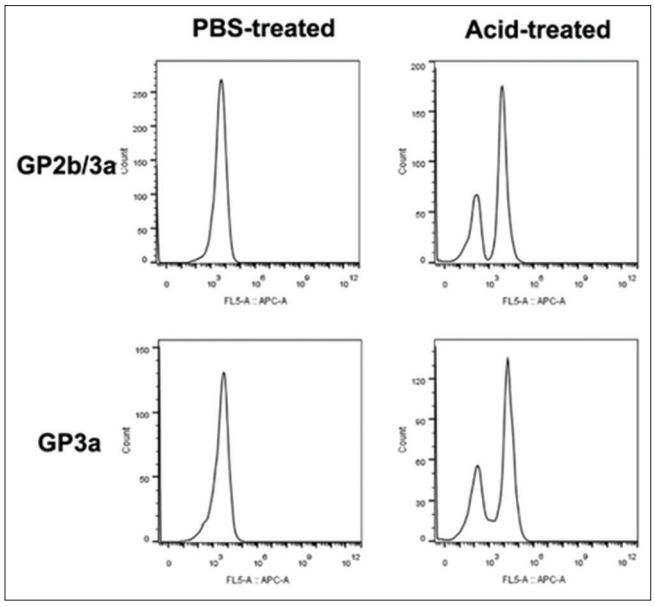

Figure 2: GP2b/3a and GP3a expression of platelet before and after citric acid treatment

plasma and 07/214 (contain HLA-I, as a weak positive control) were assigned to negative and positive controls, respectively. Figure 3 shows that a small basal increase in the acid group agreed with other controls. AB plasma after citric acid treatment showed a basal level than the untreated groups. The fluorescence value of standard serum was decreased along with the gradients. The mean fluorescence intensity (MFI) of weak control decreased proved that the HLA-I complex was broken by citric acid ($P < 0.05$, $n = 6$).

Discussion

HPA-1a is the most frequently detected antibody with specificity, which is implicated in clinically diagnosed cases, such as neonatal alloimmune thrombocytopenia, PLT transfusion refractoriness, and posttransfusion purple. The recipients may produce antibodies against HPA if the mismatched blood component is accepted. The adverse reactions caused by HPA antibodies accounted for 20%–30%, which are more severe than HLA antibodies. The specific detection of HPA antibodies is essential for preventing transfusion reaction.

Majority of immunized individuals produce HLA antibodies in addition to HPA antibodies, enhancing the difficulty in detecting HPA antibodies. Literature have reported that citric acid at pH 3.0 deleted HLA-I without damaging antigens and glycoproteins on PLTs.[4] Some studies have investigated its application in the treatment of immune thrombocytopenia but rarely in the detection of HPA antibodies. This article explored the feasibility of acid elution in testing HPA antibodies. According to acid-treated temperature, the experiment was divided

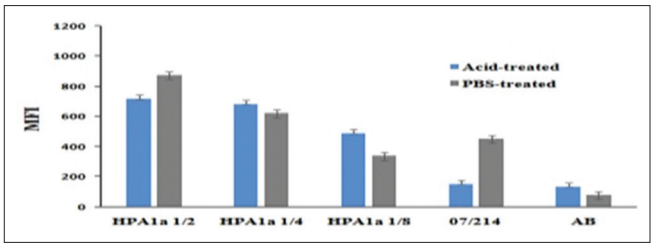

Figure 3: Fluorescence changes of human platelet antigen-1a, 07/214, and AB before and after citric acid treatment. The standard serum was diluted with corresponding AB plasma ($n = 6$, 2X ± standard deviation)

into three groups: 4°C, RT, and on -ice. Furthermore, the residue of HLA was tested based on the acid treatment time. The results showed no significant differences at 4°C and RT, but the PLTs were destroyed on ice. Damaged PLTs increased the background value of fluorescence detection. Therefore, acid buffer precooled at 4°C has been used for treating PLTs for 5 min. Figure 1 shows no statistically significant difference before and after acid treatment for CD61 on the PLTs, suggested that acid treatment was gentle for PLTs, and not damage PLTs. The method had an effect on the expression of CD62P after acid treatment, which caused an increase of CD62P. Besides, the HLA-I was removed more than 95% by exposing to low pH. It proved that the HLA-I complex was disaggregated following acid treatment.

Glycoproteins on PLTs participate in adhesion, aggregation, and releasing processes.[5] The glycoproteins that carry HPAs are considered as the target of antibodies in immunized individuals. The results showed that the expression of glycoproteins was used to explore a low pH change. The treated sample had one more peak on the left, showing that acid treatment had an effect on the expression. However, the influence was small and did not interfere with testing antibodies as the titer was high enough.

In this study, the standard serum was equal volume diluted starting with a ratio of AB-HPA-1a of 1:1 (data not shown). The MFI of HPA-1a antibodies showed no statistical differences before and after acid treatment as AB plasma-to-HPA-1a antibody ratio ranged from 1/2 to 1/8, meaning that acid treatment did not destroy the HPA-1a antigen on PLTs. The protein nonspecific adsorption on PLTs was enhanced when the background level of AB plasma was exposed to low pH, resulting in low sensitivity, compared with 1 × PBS as a negative control in which MFI would nearly be neglected. It proved that when the concentration of antibodies was high enough, then the background level would be neglected.

Conclusion

We demonstrated a simple method to screen HPA antibodies by reducing the antigenicity of HLA. However, the sensitivity needs to be improved.

Financial support and sponsorship

Written informed consent was provided, and the work has been approved by the Medical Ethics Committee of Shanghai Blood Center. This article was supported by Weigao Fund of Chinese Society of Blood Transfusion, China (Grant No.: CSBT-WG-2018-01).

Conflicts of interest

There are no conflicts of interest.

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