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Research paper

Sensitive detection of platelet-specific antibodies with a modified MAIPA using biotinylated antibodies and streptavidin-coated beads

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ABSTRACT

We have developed a modified monoclonal antibody immobilization of platelet antigens assay (MAIPA) with enhanced sensitivity in detecting antibodies against human platelet antigens (HPA), using biotinylated monoclonal antibodies, streptavidin-coated beads and detection by flow cytometry. The beads-MAIPA gave superior signal-to-noise resolution (>10-fold higher) for detection of anti-HPA-1a and anti-HPA-5b compared with the in-house standard MAIPA. Also, efficient and reproducible detection of anti-HPA-15 (CD109) was shown. The enhanced sensitivity was confirmed using WHO International Reference Reagents for anti-HPA-1a, anti-HPA-3a and anti-HPA-5b, which allowed comparison of detection endpoints with other laboratories. Finally, the beads-MAIPA was validated for quantification of anti-HPA-1a. The lower limit of quantification was 0.4 IU/mL for beads-MAIPA, compared to 1 IU/mL previously reported for standard MAIPA. Based on improved performance against all HPA-antibodies tested, the beads-MAIPA has replaced the standard MAIPA in our laboratory in diagnostics of conditions due to HPA-immunization, such as fetal and neonatal alloimmune thrombocytopenia (FNAIT).

1. Introduction

Immunization against human platelet antigens (HPA) can occur during pregnancy and after blood transfusion (Kjeldsen-Kragh and Skogen, 2013). HPA-antibodies during pregnancy may cause FNAIT, with symptoms ranging from asymptomatic thrombocytopenia to severe bleeding in the fetus or newborn. The incidence of FNAIT is reported to be 1 per 2000 live born neonates and 10–15% of these suffer from intracranial hemorrhage (Kamphuis et al., 2010). Antibodies directed against HPA-1a antigen cause the majority of FNAIT cases followed by anti-HPA-5b, but severe cases due to other HPA-antibodies are also described (Kaplan et al., 1991; Ertel et al., 2005).

Conditions due to HPA-antibodies are diagnosed in a few reference laboratories only. The reasons for this is that the methods for detection and identification of HPA-antibodies are complex and HPA-typed test platelets are required. In addition, the immunogenic epitopes are expressed on functional platelet receptors with varying expression levels, due to both individual differences and platelet activation. To detect antibodies against antigens of the HPA-3 and HPA-15 systems, fresh platelets and assays with high sensitivity are required (Berry et al., 2000; Socher et al., 2008).

The monoclonal antibody immobilization of platelet antigens assay (MAIPA) is the gold standard in detecting platelet-specific antibodies (Kiefel et al., 1987; Sachs et al., 2012). However, detection of anti-HPA-15 (CD109) has proven difficult, primarily due to low and variable expression of the antigen (Maslanka et al., 2012). Also, despite being superior to other methods, the sensitivity of the MAIPA would benefit from being increased to maximize the likelihood that all clinically relevant anti-HPA-antibodies are properly diagnosed. In particular antibodies against HPA-1a are important to monitor in affected pregnancies. In a proportion of cases, these antibodies are not detectable with standard techniques, the clinical relevance of which is still to be determined (Peterson et al., 2013).

The use of biotin and streptavidin increase sensitivity of immunoassays, both due the strong affinity as well as due to amplification of signal since each streptavidin-molecule is able to bind 4 biotin-molecules (Green, 1990; Kiefel et al., 1996), and many monoclonal antibodies are commercially available as biotin conjugates. In addition, detection of fluorescent conjugates by flow cytometry allows

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for a larger dynamic range compared with colorimetric detection using a spectrophotometer. We have developed a flow cytometry-based MAIPA using streptavidin-coated beads to capture platelet glycoproteins, followed by detection of patient antibodies using PE-conjugated secondary antibodies. The aim of the present study was to compare the sensitivity of the new beads-MAIPA with the standard MAIPA for detection of antibodies against HPAs with different specificities, and evaluate the performance when used for quantification of anti-HPA-1a.

2. Materials and methods

2.1. Platelet preparation

Platelets were isolated from EDTA-blood or buffy coat by differential centrifugation. After three washes with EDTA/PBS (10 mM EDTA in PBS, pH 6.9) the platelets were resuspended to a concentration of 750×10^9/L in EDTA/PBS + 0.1% NaN_3 and stored at +4 °C for up to 2 weeks before being used in MAIPA, except for detection of anti-HPA15 when the platelets were stored for up to 4 days.

2.2. MAIPA protocol and antibodies

MAIPA was performed essentially according to Kiefel et al. (1987). The main differences were: i) incubation with tested samples and with glycoprotein (GP)-specific antibodies were performed separately ii) the reagents in enzyme-substrate reaction were anti-human IgG-HRP (109-035-008, Jackson Immunoresearch), and TMB (331176, Kem-En-Tec Nordic). The reaction was stopped with 1 M H_2SO_4 and the absorbance was measured at 450 nm, with 570 nm as reference (SpectraMax190, Molecular Devices). The cut-off for positive result was 0.19 for GPIIb/IIIa and 0.23 for GPIa/IIa and calculated as twice the mean value for the negative control from 28 assays. GP-specific antibodies used were anti-GPIIb/IIIa, clone P2, (IM0145, Beckman Coulter) or anti-GPIa/IIa (clone AK7, MCA743, AbD Serotec).

2.3. Beads-MAIPA principle

Two major differences distinguished our new beads-MAIPA from the conventional MAIPA protocol. First, the primary antibodies used to identify the specific human platelet glycoproteins are biotinylated,

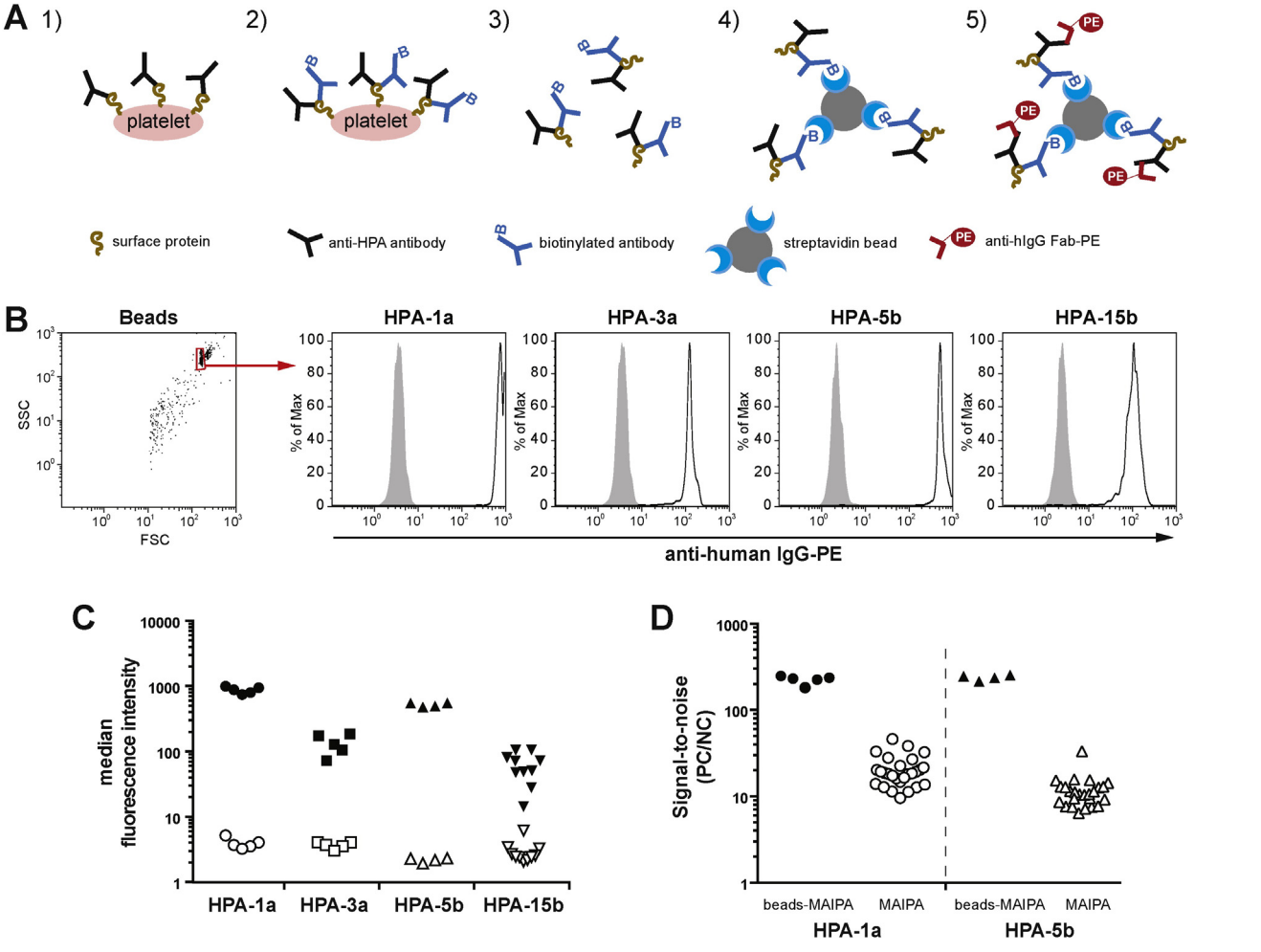

Fig. 1. a) Schematic figure of the beads-MAIPA-procedure. 1) Platelets are incubated with plasma. 2) Incubation with biotinylated glycoprotein-specific antibody. 3) Platelets are lysed. 4) Incubation with streptavidin-coated beads. 5) Incubation with anti-human IgG-PE. A wash step is performed after each incubation. Finally, the fluorescence is measured by flow cytometry, on 1000 gated beads. b) Raw data from flow cytometry analysis. Median fluorescence intensity on 1000 beads gated in FSC and SSC plot is used as read-out. Representative histograms for negative (filled) and positive (unfilled) control samples for anti-HPA-1a, anti-HPA-3a, anti-HPA-5b and anti-HPA-15b are shown. c) Sensitivity and reproducibility of beads-MAIPA. The same negative and positive samples were analyzed at different occasions with test platelets from different blood donors. Median fluorescence intensity for negative (unfilled) and positive (filled) control samples for anti-HPA-1a (HPA-1aa platelets), anti-HPA-3a (HPA-3ab or -3aa platelets), anti-HPA-5b (HPA-5ab platelets) and anti-HPA-15b (HPA-15ab and HPA-15bb platelets). d) Comparison of signal-to-noise for beads-MAIPA and MAIPA. The value for positive control divided with negative control is used to assess signal-to-noise. For beads-MAIPA the median fluorescence intensity is used as read-out and for MAIPA the optical density at 450 nm is used.

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