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Sensitive detection of idiotypic platelet-reactive alloantibodies by an electrical protein chip

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ABSTRACT

To prevent and treat immune-mediated platelet disorders (e.g. neonatal allo-immune thrombocytopenia and platelet transfusion refractoriness) the causative idiotypic platelet-reactive antibodies have to be detected with high sensitivity and specificity. The "Monoclonal Antibody Immobilization Platelet Assay" (MAIPA) is the diagnostic gold standard for immunotyping sera with respect to alloantibodies against human platelet antigens (HPA). However, it is labor-intensive and time-consuming. In this work, an automated protein chip assay (enzyme-linked sandwich immunoassay) based on interdigitated gold microelectrodes in combination with an electrical read-out system was developed and optimized. For this purpose, specific capture antibodies were immobilized on the gold electrodes. The binding of the target is detected via an enzyme-labeled detection antibody by a redox-recycling process that corresponds to the amount of bound target molecule. With this electrical chip assay it is possible to detect antibodies against HPA-1a, HPA-5b and HLA with high sensitivity and specificity in less than half the duration of the MAIPA protocol with similar intra- and interassay variance.

1. Introduction

Platelets maintain primary hemostasis. The glycocalyx layer on their surface comprises various glycoproteins. These glycoproteins mediate platelet adherence to subendothelial tissue in the injured vessel or to activated endothelial cells, as well as platelet activation after ligand binding, platelet aggregation and the formation of blood clots. Permissive point mutations in the platelet glycoprotein genes altering the molecular tertiary protein structure of these glycoproteins (Metcalfe et al., 2003) lead to a variety of polymorphic antigenic determinants on the cell surface, the human platelet specific antigens (HPA) (Newman, 1994). This can be seen as "blood groups" expressed on the platelet surface. In addition platelets also express the polymorphic human leukocyte antigen (HLA) class I system (Santoso and Kiefel, 2001). These platelet allo-antigens can induce antibodies during pregnancy or after blood transfusion. These antibodies can cause blood disorders characterized by the destruction of platelets owing to the presence of anti-platelet antibodies. One example of such diseases is the neonatal allo-immune thrombocytopenia (Kaplan, 2006), which affects about one in 1000 pregnancies (Skogen et al., 2010). Much more frequent is platelet refractoriness in patients requiring long term platelet transfusion due to chronic blood disorders. These patients depend on the availability of platelets obtained from blood donors with a compatible human platelet alloantigen pattern (Hod and Schwartz, 2008). Another rare disorder is post-transfusion purpura (Mueller-Eckhardt et al., 1980; Rozman, 2002). For all these disorders, platelet alloantibodies directed to the HPA-1 and HPA-5 system are most important in Caucasian populations. To identify affected patients and to select compatible platelets for treatment, characterization of anti-platelet antibodies is mandatory. Commercial standard diagnostics are generally enzyme-linked immunoassays, based on purified platelet glycoproteins fixed to a solid phase. The gold standard, however, is the highly sensitive, but labor-intensive and time-consuming "Monoclonal Antibody Immobilization Platelet Assay" (MAIPA) (Kiefel et al., 1987).

The aim of this study was to automate and to significantly shorten the MAIPA. Previous studies have demonstrated the feasibility of the electrical biochip technology as an alternative approach for the detection of biomolecules like DNA, rRNA, mRNA (Elsholz et al., 2009; Elsholz et al., 2006; Jürgen et al., 2005; Pioch et al., 2008) and proteins (Quiel et al., 2010). In the present study the MAIPA principle could be successfully transferred to the fully

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automated eMicroLISA biosensor device of AJ eBiochip (Itzehoe, Germany) (Albers et al., 2003) for the sensitive detection of human antibodies against HPA-1a, HPA-5b and HLA antigens, the major molecular determinants of neonatal alloimmune thrombocytopenia, within 2.5 h as opposed to the 7.5 h protocol of the standard MAIPA.

2. Materials and Methods

2.1. Sera and antibodies

Purified platelets were obtained from blood donors with blood group O who had been genotyped and phenotyped (by MAIPA) for the HPA-1 and HPA-5, and HLA class I at the Department of Immunology and Transfusion Medicine, University Greifswald, Germany. As model antigens for proof of principle, we used the clinically most important antigens, HPA-1a on the glycoprotein IIb/IIIa (fibrinogen receptor, integrin alpha 2 beta 3) and HPA-5b on the glycoprotein Ia/IIa (collagen receptor, integrin alpha 2 beta 1), as well as HLA class I antigen.

Sera containing antibodies were obtained from patients who developed these antibodies and who had been diagnosed at the Department of Immunology and Transfusion Medicine of the University of Greifswald. Three sera with different antibody patterns were used: The first serum A contained antibodies against HPA-1a, serum B against HPA-5b and serum C against HLA class I. A platelet antibody negative serum (blood group AB) served as a negative serum control. Three different monoclonal capture antibodies were used against glycoprotein IIb/IIIa (clone P2), glycoprotein Ia/IIa (clone Gi9) and HLA (clone B1G6) (Jackson ImmunoResearch Europe Ltd, Suffolk, England) and tested in the standard MAIPA in a 96-well microtiter plate format prior to the measurements with the electrical protein chip. As detection antibody for the measurements with the electrical biochip we used a polyclonal anti-human IgG antibody, labeled with biotin (Invitrogen, Carlsbad, USA).

2.2. The electrical biochip device

The electrical biochips (Hintsche et al., 1997) and the fully automated eMicroLISA device were supplied by AJ eBiochip (Itzehoe, Germany). The electrical signals were generated by amperometric redox cycling (Fig. 1) and detected by a multipotentiostat (Albers et al., 2003; Hintsche et al., 1997). The readout signals were recorded as current versus time, in nanoampere per minute (nA/min), and resulted from the sum of oxidation and reduction processes at the interdigitated array (IDA) gold electrodes. The concentration of the redox substrate p-aminophenol depended on its release from p-aminophenyl-galactopyranoside by the activity of streptavidin-ß-galactosidase whose amount reflected the level of human antibodies (Fig. 1).

2.3. Detection procedures and chip spotting

For the electrical biochip the MAIPA protocol (Kiefel et al., 1987) was modified as follows: 4×10^7 purified platelets were centrifuged at 10,000 g for one minute. The supernatant was discarded and the pellet was resuspended in 30 µL of 2% BSA (Ortho Clinical Diagnostics, Neckargemünd, Germany) diluted in PBS, pH 7.4 (Sigma Aldrich, Steinheim, Germany). The mixture was incubated with 40 µL positive or negative serum for 30 minutes at 37 °C and subsequently washed with 100 µL isotonic saline (B. Braun, Melsungen, Germany). After a centrifugation step at 10,000 g for one minute, the supernatant was discarded and the pellets were resuspended in 100 µL solubilization buffer (0.12% Tris, 0.5% Triton-X, isotonic saline solution, pH 7.4). The platelets were lysed at room temperature (RT) for 30 min and then centrifuged at 13,000 g for 20 min to remove particulate matter. 75 µL of the supernatant was diluted in 300 µL TBS wash buffer (0.12% TRIS, 0.05% Tween20, 0.5% Triton X-100, 0.05% $CaCl_2$, isotonic saline solution, pH 7.4). The lysate was used immediately or stored at −20 °C.

The used microelectrode arrays had been fabricated by photolithographic and electron-beam lithographic techniques on silicon substrates (Hintsche et al., 1997; Hintsche et al., 1994).

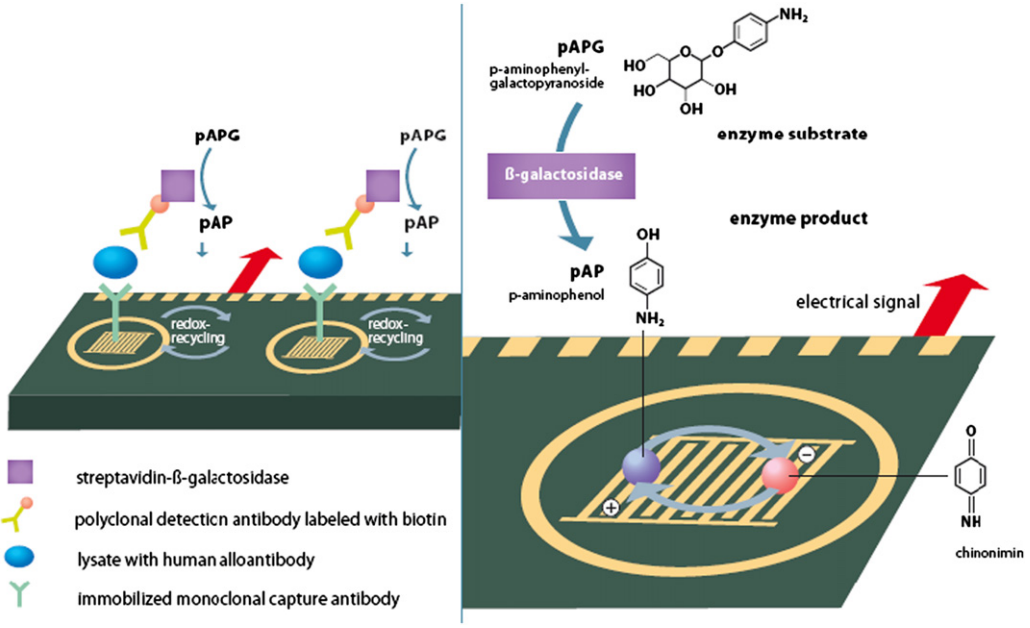

Fig. 1. Schematic presentation of the sandwich assay and the detection of the antibodies on the electrical protein chip: The capture antibody, immobilized on the gold electrodes, facilitates the binding of the target molecule in the applied sample. Afterwards, a biotin-conjugated detection antibody binds the human target alloantibody. Streptavidin-β-galactosidase then interacts with the detection antibody and causes the enzymatic release of p-aminophenol from the substrate p-aminophenyl-galactopyranoside. The subsequent redox cycling at the electrodes results in an electrical signal measured in nA/min that corresponds to the amount of bound target molecule.