Flow cytometric phenotyping of platelet HPA-1a antigen: donor screening for a case of neonatal alloimmune thrombocytopenia due to anti-HPA-1a antibodies

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Neonatal alloimmune thrombocytopenia can effectively be treated by transfusing compatible platelets to the affected newborn, but typed, compatible platelets are not generally available. For a case of probable neonatal alloimmune thrombocytopenia due to anti-HPA-1a, part of the donor population of the regional blood bank was phenotyped to find HPA-1a-negative platelets. A flow cytometric technique was used, which is reliable, rapid, and relatively simple and therefore well suited for large-scale screening. Using this method, several HPA-1a-negative donors were identified, and one of them donated platelets that were successfully transfused to the thrombocytopenic newborn. ***Immunohematology*** 1995;11:125–128.

Neonatal alloimmune thrombocytopenia (NAITP) is the platelet counterpart to hemolytic disease of the newborn (HDN), but, in contrast to HDN, NAITP frequently occurs in the first pregnancy. NAITP is caused by maternal immunization to paternal platelet antigens, which are present on fetal platelets. Platelet antibodies may cross the placenta and sensitize fetal platelets, causing thrombocytopenia in the fetal circulation. This thrombocytopenia may result in neonatal purpura or gastrointestinal or intracranial bleeding, sometimes with persistent neurological symptoms and even intrauterine hemorrhage, causing death.[1] Thrombocytopenia of a newborn infant can effectively be treated by transfusing donor platelets that do not carry the antigen to which the mother has developed antibodies.[2] Intrauterine platelet transfusions have also been shown to be effective, but this method is reserved for high-risk cases and carried out in specialized centers.[3] From the literature it is known that the bleeding risk for infants in subsequent pregnancies after a NAITP incident is at least similar to that of the previous infant, and the need for a platelet transfusion to the newborn can therefore be predicted.

At present, five platelet antigen systems are recognized,[4] of which the HPA-1 system is by far the most important for NAITP. This system comprises the antithetical antigens HPA-1a and HPA-1b. Antibodies to the HPA-1a antigen (formerly known as Zw^a and Pl^a) represent the classic example of platelet immunization causing NAITP.[5] In a large study it has been shown that over 75 percent of the cases of NAITP in a Caucasian population were due to anti-HPA-1a antibodies.[1] The frequency of the HPA-1a antigen is very high; approximately 98 percent of Caucasians are HPA-1a positive. Thus, finding compatible HPA-1a-negative donors is a difficult task for the blood bank requested to supply platelets for a delivery of a mother known to have HPA-1a antibodies. In such circumstances, the speed and reliability of flow cytometry is a reliable tool for donor phenotyping.

We present a case of a woman who had had an infant with NAITP due to anti-HPA-1a, and who was expected to deliver her second infant. The flow cytometric technique developed for screening a donor population for HPA-1a-negative platelet donors is described.

Case Report

In 1990 a 26-year-old female gave birth to her first child, who shortly after birth had a severe thrombocytopenia (10×10^9/L) with multiple petechiae. There were no other hematological abnormalities. Serologic workup in a reference laboratory revealed that the mother was HPA-1a-negative, and had HPA-1a antibodies. Both the newborn and her father were HPA-1a-positive (heterozygous); a diagnosis of NAITP due to HPA-1a antibodies was made. The newborn was treated with intravenous gamma globulin, and the platelet count rapidly returned to normal.

By the end of 1994 she was again pregnant and HPA-1a antibodies were still demonstrable in her plasma. Since the father of the second fetus was typed as homozygous for HPA-1a, it was certain that the fetus would be HPA-1a-positive. In view of the severe thrombocytopenia of the first infant, the blood bank was requested to supply compatible HPA-1a-negative platelets for the delivery. A flow cytometric technique was used to screen for HPA-1a-negative donors. After birth, the female newborn's platelet count was 17×10^9/L, and one unit of compatible, HPA-1a-negative donor platelets, found by our screening, was transfused; in addition, she was treated with intravenous gamma globulin. Immediately after transfusion, the platelet count increased to 39×10^9/L and to 115×10^9/L after 1 week. There were no signs of hemorrhage and she was discharged from the hospital in excellent health. At the age of 2 months her platelet count was 369×10^9/L.

Materials and Methods

Blood from volunteer blood bank donors was collected into K_3-EDTA and kept at ambient temperature until processing the next day. A serum containing anti-HPA-1a (Pl. 830209) was kindly provided by L. Porcelijn, MD (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), and another anti-HPA-1a serum (0193574) was from our own collection. In addition, anti-HPA-3a serum (Kl. 790411) was used. Goat anti-human IgG serum, FITC-labeled, was obtained commercially. The flow cytometer used was a FACScan (Becton-Dickinson, San Jose, CA, USA), equipped with FACScan Research software (v 2.1).

Platelet-rich plasma was prepared from EDTA-anticoagulated blood by low-speed centrifugation (180 g for 5 minutes at ambient temperature), and the platelets were washed × 3 with phosphate-buffered saline supplemented with EDTA (PBS-E). Finally, the platelets were suspended into PBS-E, giving a final concentration of @ $150 \times 10^9/\mu$L. Fifty μL of this platelet suspension were incubated at ambient temperature for 30 minutes with 75 μL of a 1:20 dilution of an anti-HPA-1a serum and then washed 3 with PBS-E; the platelets were resuspended in 100μL PBS-E and stained for 30 minutes with FITC-labeled goat anti-human IgG serum. After addition of 300 μL PBS-E, the platelet suspension was measured in the flow cytometer. First, the platelets were characterized by their forward and sideward light scatter in a two-dimensional dot plot (Fig. 1), and the subsequent fluorescence analysis was confined to these cells by means of electronic gating. Because the light scatter properties of platelets are more or less constant between normal individuals, the same electronic gating was used for an entire series of samples. In a separate experiment it had been shown, using platelet-specific monoclonal antibodies, that over 98 percent of cells within this electronic gate were indeed platelets. At least 5,000 cells were analyzed within this gate, and their FITC signal was plotted in a histogram using a logarithmic scale (Fig. 2). Platelets from

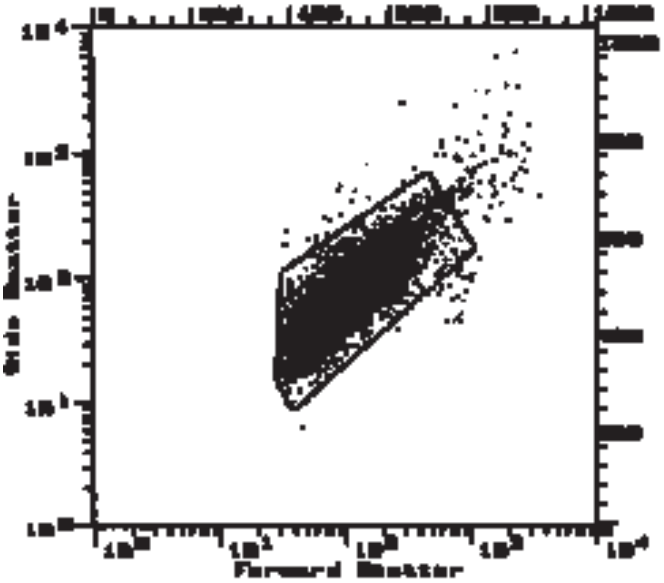

Figure 1. Dot plot of forward scatter (x axis) versus sideward scatter (y axis) of cells isolated from platelet-rich plasma and analyzed in the flow cytometer.

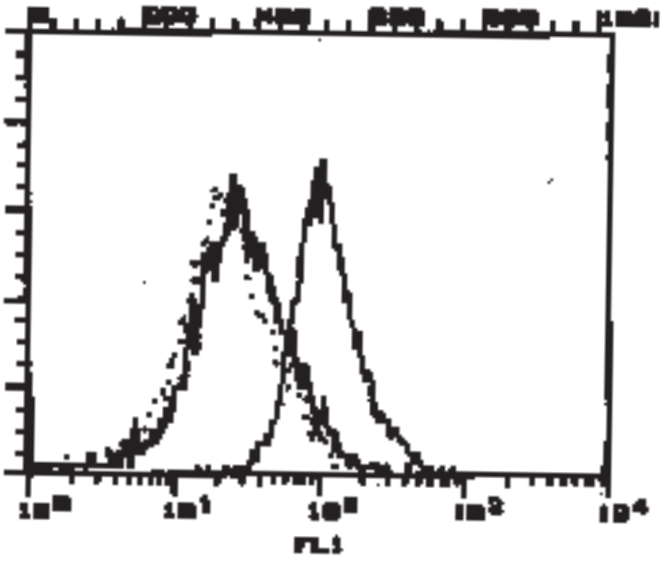

Figure 2. Overlay-histograms of HPA-1a-negative (spaced dots) and HPA-1a-positive platelets (close dots) and negative control platelets, incubated with AB serum without detectable antibodies (solid line).

two donors known to be HPA-1a-negative were run in parallel as controls.

In the screening phase, all samples from which at least 80 percent of the platelets had a FITC-fluorescence greater than the median of the HPA-1a-negative controls were regarded as probably HPA-1a-positive and were not further tested. Platelets scoring less than 80 percent relative to the median of the reference were regarded as possibly HPA-1a-negative, and an independent blood sample was obtained from these donors for confirmation testing. This confirmation testing was performed using two different anti-HPA-1a antisera. Donor platelets were designated HPA-1a-negative if the histograms obtained with the two typing sera coincided with that of the HPA-1a-negative reference platelets and to that of a negative control, which consisted of the donor's own platelets incubated with AB serum not containing platelet antibodies (Fig. 2). In addition, all HPA-1a-negative donors were typed for their HPA-3a antigen, using a single specific antiserum.

Results

Figure 1 depicts the forward and sideward light-scatter dot plot of platelets isolated from platelet-rich plasma; the cells within the polygon contained >99 percent platelets, as determined using the platelet-specific monoclonal antibody CD61 (not shown). The fluorescence signal of these platelets is shown in an overlay-histogram format: the negative control is shown, along with HPA-1a-negative and HPA-1a-positive platelets, respectively (Fig. 2).

Blood samples from 321 donors were tested; initially, eight of them (2.5%) were found in the screening phase as possibly HPA-1a-negative, but two of these donors were not available for confirmatory tests. The remaining six donors were confirmed to be HPA-1a-negative; three of them were also HPA-3a-negative and the other three HPA-3a-positive. Therefore, the frequency of HPA-1a-negative donors in our population, which is essentially completely Caucasian, was approximately 2 percent (between 1.9% and 2.5%).

Discussion

Originally, phenotyping of platelet antigens was performed using platelet agglutination and complement fixation tests, followed later by the indirect radioactive platelet Coombs test and the platelet immunofluorescence test (PIFT).[6] More recently, enzyme-linked immunoassays and solid-phase techniques have been developed for detecting platelet-bound antibodies.[2] The PIFT is most frequently used, but its main disadvantage is the microscopic estimation of the fluorescence. When the PIFT is performed using a flow cytometer, a more reproducible and objective measurement of the fluorescence signal is possible. Moreover, flow cytometry can be used for quantitative evaluation of the fluorescence intensity.

Our flow cytometric technique in its screening phase easily discriminated HPa-1a-positive subjects from possibly HPA-1a-negative ones (Fig. 2). This was demonstrated by the confirmation phase, which corroborated that all possible HPA-1a- negative donors were indeed negative when tested under more stringent conditions, including two different antisera and appropriate negative controls. Furthermore, the fact that the frequency of HPA-1a-negative subjects was exactly as can be expected in a Caucasian population generated more confidence in our technique.

Recently, Forsberg and co-workers[7] described their method for flow cytometric phenotyping of HPA-1a, which is very similar to the one described here. They found that the flow cytometric method conferred complete concordance with DNA typing of the genetic polymorphism that causes the HPA-1a and HPA-1b phenotypes.

In conclusion, as depicted by our case study, we have shown that flow cytometry offers a reliable and easy-to-handle method for large-scale HPA-1a screening and subsequent confirmation. The application described here affirms the value of flow cytometry in blood transfusion.[8]

Acknowledgments

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References

1. Mueller-Eckhardt C, Kiefel V, Grubert A, et al. 348 cases of suspected neonatal alloimmune thrombocytopenia. Lancet 1989;i:363–6.
2. Reznikoff-Etievant MF. Management of alloimmune neonatal and antenatal thrombocytopenia. Vox Sang 1988;55:193–201.
3. Lynch L, Bussel JB, McFarland JG, Chitkara U, Berkowitz RL. Antenatal treatment of alloimmune thrombocytopenia. Obstet Gynecol 1992;80:67–71.
4. ICSH/ISBT working party on platelet serology: nomenclature of platelet-specific antigens. Vox Sang 1990;58:176.

5. Blanchette VS, Chen L, Salomon de Friedberg Z, Hogan VA, Trudel E, Décary F. Alloimmunization to the Pl^{A1} platelet antigen: results of a prospective study. Br J Haematol 1990;74:209–15.
6. von dem Borne AEGKr, Verheugt FWA, Oosterhof F, Riesz E von, Brutel de la Rivière A, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol 1978;39:195–207.
7. Forsberg B, Jacobsson S, Stockelberg D, Kutti J, Rydberg L, Wadenvik H. The platelet-specific alloantigen Pl^{A1} (HPA-1a): comparison of flow cytometric immunophenotyping and genotyping using polymerase chain reaction and restriction fragment length polymorphism in a Swedish blood donor population. Transfusion 1995;35:241–6.
8. Garratty G, Arndt P. Applications of cytofluorometry to transfusion science. Transfusion 1995;35:157–78.

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