Med J Indones, Vol. 23, No. 3,
August 2014

**Clinical Research**

# Platelet reactive alloantibodies responsible for immune thrombocytopenia in Malay population

Mohd I. Armawai,[1] Afifah Hassan,[1] Hevi Wihadmadyatami,[2,3] Sentot Santoso[2]

[1] *Division of Immunohematology, National Blood Centre of Malaysia, Malaysia*

[2] *Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany*

[3] *Department of Anatomy, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia*

**Abstrak**

***Latar belakang:*** *Aloantibodi terhadap* human platelet alloantigens *(HPAs) berperan dalam terbentuknya* platelet transfusion refractoriness *(PTR) pada pasien yang mendapat transfusi trombosit dan gangguan pembekuan darah pada bayi dengan trombositopenia aloimun fetalis (FNAIT). Hasil penelitian kami sebelumnya, berdasarkan analisis distribusi alel HPAs, mengindikasikan imunisasi mungkin terjadi antara orang Melayu. Pada penelitian ini, kami mencoba untuk menganalisis frekuensi aloantibodi reaktif trombosit yang berperan dalam FNAIT dan PTR di Malaysia.*

***Metode:*** *Serum dari pasien diduga FNAIT (n = 295) dan PTR (n = 74) dikumpulkan selama lima tahun (2008-2013) dan diuji untuk mengetahui adanya antibodi platelet reaktif dengan pemeriksaan penangkapan antigen.*

***Hasil:*** *Pada 5/74 (5,41%), antibodi platelet spesifik terhadap HPA-2b (n = 1), HPA-5a (n = 1), HPA-5b (n = 1), HPA-15b (n = 2) dapat diidentifikasi dalam kelompok kohort PTR. Dalam kohort FNAIT, aloantbodi spesifik trombosit dapat dideteksi pada 18 serum (6,10%) yang terdiri atas anti-HPA-1a (n = 1), anti-HPA-3a (n = 3), anti-HPA-5a (n = 6) , anti-HPA-5b (n = 6), anti-HPA-15a (n = 1), dan anti-HPA-15b (n = 1).*

***Kesimpulan:*** *Penelitian ini memperlihatkan bahwa antibodi anti-HPA-3, -HPA-5 dan -HPA-15 merupakan antibodi spesifik trombosit yang terbanyak dalam kasus FNAIT dan PTR di populasi Malaysia. Karena distribusi alel HPA yang sama terjadi antara populasi Malaysia dan Indonesia, imunisasi terhadap tiga sistem HPA ini diperkirakan menjadi risiko paling potensial untuk gangguan trombosit yang dimediasi aloimun di Indonesia.*

**Abstract**

**Background:** Alloantibodies against human platelet alloantigens (HPAs) are responsible for the development of platelet transfusion refractoriness (PTR) in patients receiving random platelets and bleeding disorder in babies with fetal neonatal alloimmune thrombocytopenia (FNAIT). Recently, our results based on the analysis of the allelic distribution of HPAs indicated that immunization may occur among Malay. In this study, we sought to analyze the frequencies of platelet reactive alloantibodies responsible for FNAIT and PTR in Malaysia.

**Methods:** Sera from suspected FNAIT (n = 295) and PTR (n = 74) were collected in five years period (2008-2013) and tested for the presence of platelet reactive antibodies by the use of antigen capture assay.

**Results:** In 5/74 (5.41%) platelet specific antibodies against HPA-2b (n = 1), HPA-5a (n = 1), HPA-5b (n = 1), HPA-15b (n = 2) could be identified in our PTR cohort. In FNAIT cohort, platelet specific alloantibodies could be detected in 18 sera (6.10%) consisting anti-HPA-1a (n = 1), anti-HPA-3a (n = 3), anti-HPA-5a (n = 6), anti-HPA-5b (n = 6), anti-HPA-15a (n = 1), and anti-HPA-15b (n = 1).

**Conclusion:** Our study indicates that anti-HPA-3, -HPA-5 and -HPA-15 antibodies seems to be the most platelet specific antibodies involved in FNAIT and PTR cases in Malaysian population. Since similar HPA allelic distribution among Malaysian and Indonesian populations have been observed, immunization against these three HPA systems are expected to be the most potential risk of alloimmune mediated platelet disorders in Indonesia.

**Keywords:** alloimmune thrombocytopenia, transfusion

pISSN: 0853-1773 • eISSN: 2252-8083 • http://dx.doi.org/10.13181/mji.v23i3.1011 • Med J Indones. 2014;23:158-62

Correspondence author: Sentot Santoso, sentot.santoso@immunologie.med.uni-giessen.de

Human platelet alloantigens (HPAs) are immunogenic polymorphic forms of platelet membrane glycoproteins (GPs) GPIa, GPIb, GPIIb, GPIIIa and CD109. Meanwhile, 28 HPA biallelic systems have been discovered, all of which except one are defined by single amino substitution, caused by single nucleotide polymorphism (SNP) in the respective gene;[1] (see official homepage http://www.ebi.ac.uk/ipd/hpa/for recent update).

Alloantibodies against HPAs are involved in three major clinical syndromes: fetal neonatal alloimmune thrombocytopenia (FNAIT), platelet transfusion refractoriness (PTR), and post transfusion refractoriness (PTP). In addition, recent evidence indicated that antibodies against HPAs are also importance in transplantation settings.[2]

FNAIT is caused by HPA incompatibility between mother and fetus. During the pregnancy, maternal alloantibodies cross the placenta and mediate the destruction of fetal platelets. In Caucasian, anti-HPA-1a is the most commonly involved in FNAIT (75%), followed by anti-HPA-5b antibodies.[3] In PTR cases, patients fail to respond adequately to an appropriate dose of platelet transfusion. The immune-related PTR is mostly caused by antibodies against HLA class I antigens. However, alloantibodies against HPAs have been observed in 20-30% of PTR cases, mostly against HPA-1b and HPA-5b.[4] PTP is a rare transfusion reaction characterized by sudden onset of thrombocytopenia. The antibodies (mostly anti-HPA-1a) present in the recipient cause not only the destruction of donor's platelets but also autologous platelets. The mechanism underlying this phenomenon, however, is still unclear.[5]

Presently, the diagnosis of alloimmune mediated thrombocytopenia relies on the assignment of HPA incompatibility and on the identification of anti-HPA alloantibodies. Based on the underlying SNPs, several molecular biological methods have been established in the last decade, which now allow reliable high-resolution and high-throughout genotyping analysis of HPAs.[6] On account of these technologies, extensive studies on the gene frequency of HPAs have been conducted worldwide. However, the identification of antibodies is mandatory to define the correct clinical diagnostic and treatment strategy of patients suffering from alloimmune thrombocytopenia.

Recently, the distribution of HPA gene frequencies has been reported in Malaysia as well as Indonesia.[7,8] Until today, however, no data is available about the frequencies and the clinical relevance of HPA alloantibodies in these countries. In this study, we presented the first data according the frequencies of platelet reactive alloantibodies in Malaysia, which are responsible for FNAIT and PTR.

## METHODS

### Patients

Sera from suspected FNAIT (n = 295) and PTR (n = 74) were collected in five years period (2008-2013). Incompatibility on HPA systems was verified by genotyping of maternal/paternal or donor/recipient DNA. Genomic DNA was isolated from the leukocytes samples, and then genotyped by the allele-specific PCR as recommended by the manufactures (GTI, San Diego, CA, USA and BAGene, Lich, Germany). PCR products were analyzed by 2% agarose gel electrophoresis and were visualized using a UV transilluminator. Informed consent was obtained from all subjects and the study was approved by the institution's review board.

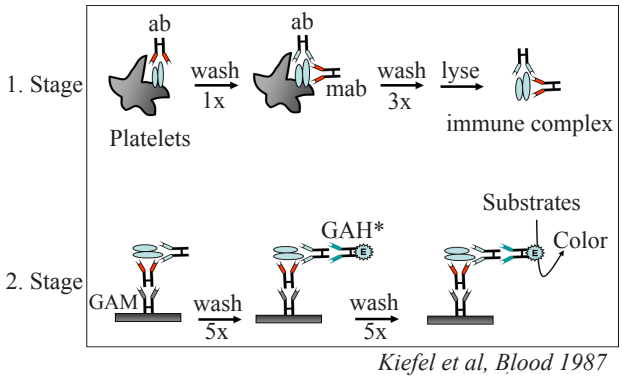
### Monoclonal antibodies (Mabs)

Mabs against glycoprotein (GP) IIb/IIIa (clone Y2/51; DakoCytomation; Denmark), GPIb/IX (clone SZ2; Beckman Coulter, Marseille, France), GPIa/IIa (clone Gi9; Beckman Coulter, Marseille, France), CD109 (clone TEA/16; BD Pharmingen, USA), and specific for HLA Class I (clone B.9.12.1) were used for the antigen capture assay (see below).

### Detection of platelet alloantibodies by antigen capture assay

Alloantibodies were analyzed using a panel of known HPAs typed platelets (HPA-1,-2,-3,-5, and -15) from blood group O and paternal platelets by antigen capture assay, MAIPA (monoclonal antibody immobilized platelet antigen) as previously described.[9,10] The principle of this assay is illustrated in figure 1.

In brief, $100x10^9$/L washed platelets of HPA phenotyped donors were incubated with 25µL of human serum for 30 min at 37°C. After washings with 200 µL TBS/BSA buffer (0.099M Tris buffered saline containing 0.2% bovine serum albumin), sensitized platelets were incubated with mabs for 30 min at 37°C and washed again 3 times with TBS/




*Kiefel et al, Blood 1987*

**Figure 1.** The priciple of the MAIPA assay

BSA buffer. Subsequently, platelets were solubilized for 15 min at room temperature (RT) with 130µL solubilization buffer (TBS containing 0.5% Triton X-100) prior to centrifugation for 15 min at RT (13,000 g). The supernatants were then transferred to an F-microtiter plate coated with goat anti-mouse IgG (dilution 1:500; Jackson Immunoresearch, Pennsylvania, USA). After incubation (30 min at 37°C) the wells are tipped out and washed 5 times with 125µL washing buffer (0.099M TBS containing 0.5% NP40 and 0.2% Tween 20), and incubated with 100µL of peroxidase conjugated goat anti-human IgG (Jackson Immunoresearch; dilution 1/6000) for 1 h at RT. Finally, the wells were washed 5 times with 125µL washing buffer. The reaction was visualized with 100uL of ortho-pheneylendiamin (Dako) substrate solution (15 min in the dark at RT), stopped by adding 100µL 0.5M $H_2SO_4$, and measured in photometer at 490nm (Dynex Technologies Inc., Virginia, USA).

## RESULTS

In total of 295 samples of suspected FNAIT cases, platelet antibodies against HPAs were found in 18 sera (6.10%) by the MAIPA which consist of alloantibodies against HPA-1a (n = 1), HPA-3a (n = 3), HPA-5a (n = 6), HPA-5b (n = 6), HPA-15a (n = 1), and HPA-15b (n = 1). In addition, platelet reactive antibodies against HLA class I molecule could be detected in 58 samples (19.67%) (Table 1). Current evidence indicate that anti-HLA class I antibodies do not seem to play a major role on the mechanism of FNAIT. However, limited cases of FNAIT associated with HLA class I antibodies have been described.[11] In PTR cases, platelet specific antibodies against HPAs were detected in 4/74 cases only (5%). Anti-HPA-2b (n = 1), anti-HPA-5a (n = 1), anti-HPA-5b (n = 2), anti-HPA-15b (n = 3) could also be identified. In addition, we also found pan-reactive antibodies against GPIIb/IIIa (n = 11) and GPIb/IX (n = 1) in 16.2% of PTR cases.

Table 2 shows the allelic distribution of HPA systems in Malaysia and Indonesia. No significant different between these two populations could be observed. The HPA-1b allele frequency among Malay (both in Malaysia and Indonesia) was significantly lower than the frequency reported among Caucasian population (0.024 versus ~0.133). Thus, in contrast to Caucasian, alloimmune mediated thrombocytopenia caused by anti-HPA-1a antibodies seems to be extremely rare occurrence among Malay ethnics. However, we found a case of FNAIT associated with anti-HPA-1a antibodies. Furthermore, different allelic

**Table 1.** The frequencies of platelet antibodies found in suspected FNAIT and PTR cases

| Alloantibody specificity | FNAIT (n = 295) | PTR (n = 74) |
|---|---|---|
| HLA class I | 58 | 24 |
| HPA-1a | 1 | - |
| HPA-2b | - | 1 |
| HPA-3a | 3 | - |
| HPA-5a | 6 | 1 |
| HPA-5b | 6 | 1 |
| HPA-15a | 1 | - |
| HPA-15b | 1 | 2 |
| GPIIb/IIIa | - | 11 |
| GPIb/IX | - | 1 |

**Table 2.** Gene frequencies of HPA-1 to HPA-5 and HPA-15 in Malaysia (n = 200) and Indonesia. (n = 500) (Tan et al, 2012; Asmarinah et al 2013)

| HPA system | Gene frequency Malaysia | Gene frequency Indonesia | Gene frequency Caucasian |
|---|---|---|---|
| HPA-1a | 0.975 | 0.977 | 0.877 |
| HPA-1b | 0.025 | 0.023 | 0.133 |
| HPA-2a | 0.962 | 0.940 | 0.943 |
| HPA-2b | 0.038 | 0.060 | 0.057 |
| HPA-3a | 0.502 | 0.507 | 0.471 |
| HPA-3b | 0.498 | 0.493 | 0.529 |
| HPA-4a | 0.995 | 0.948 | 1.000 |
| HPA-4b | 0.005 | 0.052 | 0.000 |
| HPA-5a | 0.950 | 0.968 | 0.929 |
| HPA-5b | 0.050 | 0.032 | 0.071 |
| HPA-15a | 0.515 | 0.551 | 0.495 |
| HPA-15b | 0.485 | 0.449 | 0.505 |

distribution was observed for HPA-3 and HPA-15 systems. In Malays, HPA-3a and HPA-15a represent the frequent allele whereas in Caucasian population is contrariwise. Since HPA-3a/HPA-3b and HPA-15a/HPA-15b alleles are equally distributed, alloimmunization against these HPA systems is expected.

## DISCUSSION

This study was aimed to evaluate the clinical impact of platelet reactive alloantibodies responsible for the pathomechanism of FNAIT and PTR cases in Malaysia. High prevalence of anti-HPA-3a, anti-HPA-5a, and anti-HPA-5b alloantibodies was found in our FNAIT cases. In this cohort, anti-HPA-3a alloantibodies were associated with severe thrombocytopenia (platelet count < $50x10^9$/L) in the newborns. This is in accordance with the current data reported by the Japanese Society on Platelet Serology.[12] All of the neonates was admitted to the NICU (Neonatal Intensive Care Unit) for close monitoring and received intravenous gamma-globulin (IVIG; 0.4g/kg body weight over five days) as recommended.[13] Based on the delay of IVIG response (24-48 hours), neonates were transfused with mother's platelets (HPA-3a negative) in order to maintain thrombocytopenia and bleeding tendency in these babies. Unfortunately, more improved therapy regiment by transfusion with HPA-3 negative platelets from healthy individuals was not possible due to a limited register of HPA typed donors in our National Blood Centre in Kuala Lumpur. To prevent long-term disability or death of neonates caused by intracranial hemorrhage during the pregnancy in suspected severe FNAIT cases, early identification of anti-HPA-3a alloantibodies is recommended.[14] In accordance to previous observation, only mild thrombocytopenia was observed in all FNAIT cases associated with anti-HPA-5a and -5b.[15]

In our PTP cohort, we found high prevalence of anti-HPA-5b and anti-HPA-15b alloantibodies, and quite high number of pan-reactive antibodies (without allele specificity) against platelet GPIIb/IIIa and GPIb/IX. The question whether this pan-reactive antibodies represents autoantibodies are difficult to answer, because we are unable to perform direct antibody testing on patient's platelet due to limited amount of platelets.

Based on the similar HPA allelic distribution among Malaysian and Indonesian population, immunization against HPA-3, -5 and -15 systems are expected to be the most potential risk for the development of FNAIT and PTR among Indonesian population. Therefore, screening of platelet antibodies against these three HPA systems should be recommended for the diagnosis of FNAIT and PTR. In antibody positive patients, HPA-compatible platelet transfusion should be given to improve the treatment of these disorders. Beside HPA-compatible platelets, however, compatibility between HLA antigens should also take into account when anti-HLA class I antibodies are also found. Providing HPA and HLA compatible platelets for patient's in-need is challenging task. Therefore, typing of many donors for these antigen systems and performing national donor registry is mandatory to solve this problem.

In conclusion, our study indicates that anti-HPA-3, -5 and -15 antibodies seems to be the most clinically important platelet specific antibodies involved in FNAIT and PTR cases in Malaysian population. Because of the lability of the HPA-3 and HPA-15 antigenic structures located on GPIIb and CD109, respectively, the detection of anti-HPA-3 and anti-HPA-15 alloantibodies is challenging and therefore some of the cases may overlook by the current assays. The use of fresh platelets is recommended to enhance the sensitivity of platelet antibody detection.[16-18]

### Conflict of interest

All authors have nothing to disclose.

## REFERENCES

1. Metcalfe P, Watkins NA, Ouwehand WH, Kaplan C, Newman P, Kekomaki R, et al. Nomenclature of human platelet antigens. Vox Sang. 2003;85(3):240-5.
2. Juji T, Watanabe Y, Ishikawa Y, Fujiwara K, Tonami H, Tanaka H, et al. Human platelet alloantigen (HPA)-5a/b mismatch decreases disease-free survival in unrelated bone marrow transplantation. Tissue Antigens. 1999;54(3):229-34.
3. Mueller-Eckhardt C, Grubert A, Weisheit M, Mueller-Eckhardt G, Kiefel V, Kroll H, et al. 348 cases of suspected neonatal alloimmune thrombocytopenia. Lancet. 1989;333(8634):363-6.
4. Kiefel V, Konig C, Kroll H, Santoso S. Platelet antibodies in transfused patients. Transfusion. 2001;41(6):766-70.
5. Kiefel V, Schönberner-Richter I, Schilf K. Anti-HPA-1a in a case of post-transfusion purpura: binding to antigen-negative platelets detected by adsorption/elution. Transfusion Med. 2005;15(3):243-7.
6. Veldhuisen B, Porcelijn L, Ellen van der Schoot C, de Haas M. Molecular typing of human platelet and

neutrophil antigens (HPA and HNA). Transfus Apher Sci. 2014;50(2):189-99.
7. Tan J.Y, Lian L.H, Nadarajan V.S. Genetic polymorphisms of human platelet antigen-1 to -6 and -15 in the Malaysian population. Blood Transfusion. 2012;10(3):368-76.
8. Asmarinah, Dharma R, Ritchie N.K, Rahayu S, Putricahya E, Santoso S. Human platelet-specific antigen frequencies in Indonesian population. Transfus Med. 2013;23(4):250-3.
9. Kiefel V, Santoso S, Weisheit M, Müeller-Eckhardt C. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood. 1987;70(6):1722-6.
10. Campbell K, Rishi K, Howkins G, Gilby D, Mushens R, Ghevaert C, et al. A modified fast MAIPA for the detection of HPA antibodies: a multi-centre evaluation of a rapid monoclonal antibody specific immobilisation of platelet antigen (MAIPA) assay. Vox Sang. 2007;93: 289-97.
11. Saito S, Ota M, Komatsu Y, Ota S, Aoki S, Koike K, et al. Serologic analysis of three cases of neonatal alloimmune thrombocytopenia associated with HLA antibodies. Transfusion. 2003;43(7):908-17.
12. Tsuno NH, Matsuhashi M, Iino J, Nagura Y, Okazaki H, Santoso S. The importance of platelet antigens and antibodies in immune-mediated thrombocytopenia. ISBT Science Series. 2014;9(1):104-11.
13. Sidiropoulos D, Straume B. The treatment of neonatal isoimmune thrombocytopenia with intravenous immunoglobulin. Blut. 1984;48:383-56.
14. Roberts I, Stanworth S, Murray NA. Thrombocytopenia in the neonates. Blood reviews. 2008;22(4):173-86.
15. Warkentin TE, Smith JW, Hayward CP, Ali AM, Kelton JG. Thrombocytopenia caused by passive transfusion of anti-glycoprotein Ia/IIa alloantibody (anti-HPA-5b). Blood. 1992;79(9):2480-4.
16. Ertel K, Al-Tawil M, Santoso S, Kroll H. Relevance of the HPA-15 (Gov) polymorphism on CD109 in alloimmune thrombocytopenia syndromes. Transfusion. 2005;45(3):366-73.
17. Socher I, Kroll H, Jorks S, Santoso S, Sachs UJH. Heparin-independent activation of platelets by heparin-induced thrombocytopenia antibodies: a common occurance. J Thromb and Haemost. 2008;6(1):197-200.
18. Matsuhashi M, Tsuno NH, Sone S, Mishima Y, Nagura Y, Watanabe-Okochi N, et al. The role of alloantibodies against human platelet antigen-15 in multiply platelet transfused patients. Transfusion. 2014;54(4):1093-9.