Quantitation of Lan antigen in Lan+, Lan+^{w} and Lan– phenotypes

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Introduction

The Lan antigen (LAN1) is a clinically significant, high-frequency red blood cell (RBC) antigen. Lan was originally described in 1961 and assigned to the LAN blood group system in 2012[1,2]. Lan is currently the only antigen within the LAN blood group system and Lan+, Lan–, Lan+^{w} and Lan+^{w}/– phenotypes have been defined[1-4].

The Lan– phenotype is very rare worldwide with a frequency of less than 1% in all populations tested to date[2,5,6]. For example, a Japanese screening study identified 14 Lan– individuals among 713,384 blood donors, giving a frequency of 0.002%[2]. Lan– individuals are usually identified due to the detection of anti-Lan during investigations into haemolytic disease of the foetus and newborn[7,8] or when serological testing is performed to find compatible blood units for a patient[5,9]. Transfusion support for Lan– individuals is highly challenging due to the scarcity of both compatible blood and suitable anti-Lan reagents for screening for compatible blood. The first monoclonal anti-Lan antibody (OSK43) was produced in 2012 by Helias *et al.* and this antibody has proven to be of huge benefit as a reliable reagent for screening for Lan– individuals[2].

The carrier of the Lan antigen is the ABCB6 protein encoded by the *ABCB6* gene at chromosome 2q36, containing 19 exons[2]. Numerous genetic variants have been identified as encoding for the Lan– phenotype[2,4,10-12]. The initial sequencing study identified ten novel alleles, including frameshift, nonsense and splice-site mutations, within 12 unrelated Lan– individuals and found that each individual was heterozygous for two *ABCB6* null alleles[2]. Subsequently, Saison *et al.* characterised yet another *ABCB6* null allele, a single nucleotide variant missense mutation (c.574C>T)[10]. To date this single nucleotide variant is the most common mutation causing the Lan– phenotype[4,10]. It has been suggested that the frequency of *ABCB6* null alleles differs between populations as sequencing of 27 Japanese Lan– individuals identified a further ten novel alleles and revealed that none of the donors carried the c.574C>T variant[12].

The Lan+^{w} phenotype has been described in individuals heterozygous for an *ABCB6* null allele and a wild-type allele, and these individuals typically express 50% of the normal level of Lan antigen[4,10]. The fourth Lan phenotype is referred to as Lan+^{w}/– as cells serologically type as either Lan+^{w} or Lan– depending on the anti-Lan utilised[3]. A comprehensive study involving serological and molecular characterisation of Lan phenotypes was recently performed by Reid *et al.* and for the first time alleles encoding Lan+^{w}/– phenotypes were defined[4]. Lan+^{w}/– individuals are heterozygous for an *ABCB6* null allele and a variant allele encoding for weakened Lan expression.

Antigens can be quantified by flow cytometry by converting the fluorescent intensity of staining into an antibody-binding capacity (ABC), relating to the number of monoclonal antibody molecules bound to a cell. This is performed utilising populations of calibrated microspheres coated with defined amounts of a capture antibody, to create a calibration curve used for quantitation of ABC. The usefulness of determining ABC values has been well established, particularly for lymphocyte antigens[13]. In previous studies, calibrated microsphere-based assays have been validated to aid in the diagnosis, prognosis, and treatment monitoring of diseases including chronic lymphocytic leukaemia[14,15] and human immunodeficiency virus infection[16]. In the context of RBCs, glycophorin A and RhD antigen expression has been investigated utilising traditional flow cytometric approaches[17,18] and, more recently, calibrated microspheres[19,20].

The variability of Lan antigen expression has never been quantitatively investigated. In this study, we investigated the expression of Lan antigen by developing a novel indirect staining protocol capable of quantitating the number of Lan sites per RBC in samples reported as Lan+, Lan+^{w}, and Lan–.

Materials and methods

Quantitation of Lan antigen sites

Lan antigen sites were quantitated by flow cytometry using calibrated microspheres (Quantum Simply Cellular, Bangs Laboratories Inc., Fishers, IN, USA). Calibrated microspheres are a mixture of four microsphere populations coated with incremental levels of IgG specific for the Fc segment of human IgG. The precise amount of bound antibody is determined by the

manufacturer to allow determination of ABC based on a 1:1 ratio of antibody to fluorophore.

To determine the ABC of cells accurately, saturating antibody conditions must be utilised to allow for fluorescent intensity to be related to the number of bound antibody molecules, equivalent to the number of antigen sites. The antibody volumes described below were determined to be saturating (*data not shown*). Microsphere fluorescence was determined using the same staining protocol utilised for RBC samples, as described below.

Red blood cell samples

Lan+^{w} and Lan– samples. Cryopreserved RBC samples, phenotyped as either Lan+^{w} (n=4) or Lan– (n=10) were recovered from the sample archives of the Australian Red Cross Blood Service Red Cell Reference Laboratory.

Lan+ samples. RBCs from three randomly selected Lan+ blood donors were included in each experiment. As the Lan+^{w} and Lan– samples available for this study were cryopreserved, aliquots of the Lan+ RBC samples were cryopreserved prior to utilisation.

Sample preparation. Cryopreserved RBC samples were thawed in Celpresol (CSL, Parkville, Victoria, Australia) warmed to 37 °C. Once thawed, RBCs were washed and prepared as 2% RBC suspensions.

Staining procedure

The Lan antigen density of RBC samples was investigated in triplicate using calibrated microspheres according to the manufacturer's instructions. Briefly, 150 µL of each 2% RBC suspension were combined with 16 µL of monoclonal IgG anti-Lan (OSK43) diluted in 84 µL phosphate-buffered saline (PBS). In parallel to RBC samples, a separate tube was used to generate a linear regression curve. For this, one drop of each microsphere population 1-4 was pooled and stained. Each tube was incubated at 37 °C for 1 hour. Following incubation, the RBCs were washed twice by addition of PBS, centrifuged at 3,000 rpm for 30 seconds and then the supernatant was aspirated. In parallel, microspheres were washed twice by addition of PBS, centrifuged at 2,500 *g* for 5 minutes and then the supernatant was aspirated. Pellets were resuspended in 5 µL of biotin-SP-conjugated AffiniPure Fab fragment goat anti-human IgG (H&L) (Cat N. 109-067-003, Jackson Immuno Research Laboratories Inc, West Grove, PA, USA) diluted in 95 µL PBS and incubated for 30 minutes, at room temperature, in the dark. Washing was performed twice as described and each pellet resuspended in 20 µL of Streptavidin PE (Cat. N. 349023, BD Biosciences, San Jose, CA, USA) diluted in 80 µL of PBS. The suspension was incubated for 30 minutes, at room temperature, in the dark. Washing was performed and pellets resuspended in 300 µL of PBS. RBCs were aspirated twice through a 23G fine needle to disaggregate RBC agglutinates. Samples were analysed using a FACS CANTO II flow cytometer and FACSDiva software (BD Biosciences). The "blank" microsphere population was also analysed to determine background fluorescence. For RBC samples, 20,000 events were acquired, and for microspheres 4,000 events were acquired.

Calculation of antigen-binding capacity values

A linear regression curve was constructed by plotting the mean fluorescence intensity of each microsphere population against the defined ABC value of the population using the QuickCal spreadsheet provided by Bangs Laboratories specifically for each lot of calibrated microspheres. The ABC of each RBC sample was calculated by comparing the mean fluorescence intensity of each sample to the curve and by assuming a 1:1 ratio of fluorescence to Lan sites per RBC.

Results

Lan antigen quantitation

Seventeen samples were analysed, including Lan+ (n=3), Lan+^{w} (n=4) and Lan– (n=10) phenotypes. The ABC values of the Lan+ donors were 859±174, 1,047±203, and 1,150±218 Lan antigen sites per RBC (Figure 1). The Lan+^{w} ABC values were 139±86, 180±36, 340±68 and 428±28 sites per RBC. The ABC values of the Lan– samples were calculated as being between 3±1 to 46±3 sites per RBC. Based on these values we concluded that an ABC value <50 was due to background fluorescence and/or non-specific binding and could be confidently called Lan–.

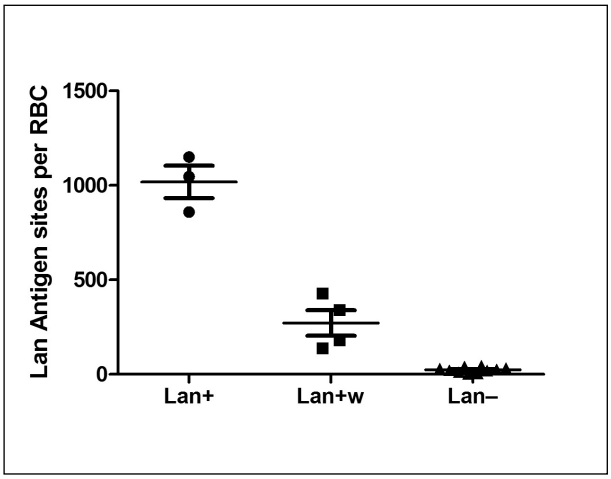

Figure 1 - Lan antigen expression of Lan+ (n=3), Lan+w (n=4) and Lan– (n=10) samples determined by a quantitative flow cytometry assay utilising calibrated microspheres.

Discussion

Conversion of mean fluorescence intensity into an ABC value allows a greater level of sensitivity in detecting quantitative differences compared to standard flow cytometry or serological techniques. Calibrated microspheres assume that antigen-antibody interaction on the RBC membrane occurs in a 1:1 ratio, meaning that at saturating conditions, each Lan site binds one anti-Lan molecule, allowing for the determination of the number of Lan sites per RBC. This study reports on the quantitation of Lan sites per RBC and the distinct levels of Lan expression within the Lan phenotypes Lan+, Lan+^{w} and Lan–.

Two of four Lan+^{w} samples investigated had ABC values around 60% lower than those of the Lan+ samples (average of 1,019 sites per RBC for Lan+ *vs* 340±68 and 428±28 for the Lan+^{w} samples). This level of antigen expression is consistent with these individuals being heterozygous for an *ABCB6* null allele and a wild-type allele. The remaining two Lan+^{w} samples had ABC values around 85% lower than those of the Lan+ samples (139±86 and 180±36). This suggests these individuals may be more accurately phenotyped as Lan+^{w}/– and this level of expression is consistent with heterozygosity for an *ABCB6* null allele and a variant allele encoding for weakened expression[4]. A limitation of this study was that no genomic DNA was available for investigation. Although the genetic basis of Lan phenotypes is too complex to allow routine genotyping, for research purposes it would have been of interest to sequence the *ABCB6* gene of our sample set to determine the genetic basis of the Lan–, Lan+^{w} and Lan+^{w}/– phenotypes.

A further limitation of this study is the small scale of testing performed. Due to the rarity of Lan–, Lan+^{w} and Lan+^{w}/– samples the capacity to perform a more comprehensive study would be restricted to large reference laboratories. Furthermore, testing multiple examples of anti-Lan would be of interest as there is the potential that this would result in the determination of different ABC values due to unique antibody specificity and affinity. However, at this time OSK43 is the only monoclonal anti-Lan available.

Conclusions

We report the development of a flow cytometry assay which shows promise in investigating the level of Lan antigen per RBC and could be used by reference laboratories as a confirmation of Lan+, Lan–, Lan+^{w} and even Lan+^{w}/– phenotypes. We believe that development of a flow cytometric assay was necessary for sensitive determination of Lan phenotypes because there are difficulties with traditional serological and genetic methods, given the rarity and variability of strength of antisera and the complex genetic basis of Lan phenotypes.

Acknowledgements

The anti-Lan (OSK43) was kindly provided by Dr Yoshihiko Tani, Japanese Red Cross Kinki Block Blood Centre, Osaka, Japan.

We thank the Alexander Steele Young Memorial Lions Foundation for scholarship support of Rhiannon McBean's PhD study.

Australian governments fully fund the Australian Red Cross Blood Service for the provision of blood products and services to the Australian community.

Authorship contributions

RM designed and performed all experiments and analysed data. BW and Y-WL provided all RBC samples, sourced the anti-Lan (OSK43), and provided expert serological advice. CH and RF supervised the study design and reviewed data. RM wrote this manuscript and all authors contributed to reviewing the manuscript.

The Authors declare no conflict of interest.

References

1) van der Hart M, Moes M, van der Veer M, van Loghem JJ. Ho and Lan- two new blood group antigens. Proceedings of the 8th Congress of the European Society of Haematology, Vienna, 1961; 493-505.
2) Helias V, Saison C, Ballif BA, et al. ABCB6 is dispensable for erythropoiesis and specifies the new blood group system Langereis. Nat Genet 2012; **44**: 170-3.
3) Storry JR, Oyen R. Variation in Lan expression. Transfusion 1999; **39**: 109.
4) Reid ME, Hue-Roye K, Huang A, et al. Alleles of the LAN blood group system: molecular and serologic investigations. Transfusion 2014; **54**: 398-404.
5) Okubo Y, Yamaguchi H, Seno T, et al. The rare red cell phenotype Lan negative in Japanese. Transfusion 1984; **24**: 534-5.
6) Daniels G. *Human Blood Groups*. 3rd ed. Oxford: Wiley-Blackwell Publishing; 2013.
7) Smith DS, Stratton F, Johnson T, et al. Haemolytic disease of the newborn caused by anti-Lan antibody. Br Med J 1969; **3**: 90-2.
8) Page PL. Hemolytic disease of the newborn due to anti-Lan. Transfusion 1983; **23**: 256-7.
9) Grindon AJ, McGinniss MH, Issitt PD, et al. A second example of anti-Lan. Vox Sang 1986; **15**: 293-6.
10) Saison C, Helias V, Peyrard T. The ABCB6 mutation p.Arg192Trp is a recessive mutation causing the Lan- blood type. Vox Sang 2013; **104**: 159-65.
11) Haer-Wigman L, Ait Soussan A, Ligthart, P, et al. Molecular analysis of immunized Jr(a–) or Lan– patients and validation of a high-throughput genotyping assay to screen blood donors for Jr(a–) and Lan– phenotypes. Transfusion 2014; **54**: 1836-46.
12) Tanaka M, Yamamuro Y, Takahashi J, et al. Novel alleles of Lan− in Japanese populations. Transfusion 2014; **54**: 1438-9.
13) Lenkei R, Andersson B. Determination of the antibody binding capacity of lymphoctye membrane antigens by flow cytometry in 58 blood donors. J Immunol Methods 1995; **183**: 267-77.

14) Rossmann E, Lenkei R, Lundin J, et al. Performance of calibration standards for antigen quantitation with flow cytometry in chronic lymphocytic leukemia. Cytometry B Clin Cytom 2007; **72**: 450-7.
15) Geisler C, Larsen J, Hansen N, et al. Prognostic importance of flow cytometric immunophenotyping of 540 consecutive patients with B-cell chronic lymphocytic leukemia. Blood 1991; **78**: 1795-802.
16) Ginaldi L, De Martinis M, D'Ostilio A, et al. Altered lymphocyte antigen expressions in HIV infection: a study by quantitative flow cytometry. Am J Clin Pathol 1997; **108**: 585-92.
17) Langlois RG, Bigbee WL, Jensen RH. Flow cytometric characterization of normal and variant cells with monoclonal antibodies specific for glycophorin A. J Immunol 1985; **134**: 4009-17.
18) Flegel WA, Curin-Serbec V, Delamaire M, et al. Section 1B: Rh flow cytometry. Coordinator's report. Rhesus index and antigen density: an analysis of the reproducibility of flow cytometric determination. Transfus Clin Biol 2002; **9**: 33-42.
19) Riquelme BD, de Isla NG, Valverde JR, Stoltz JF. A simple method for quantifying high density antigens in erythrocyte membrane by flow cytometry. J Biochem Biophys Methods 2006; **68**: 31-42.
20) McGann PT, Despotovic JM, Howard TA, Ware RE. A novel laboratory technique demonstrating the influences of RHD zygosity and the RhCcEe phenotype on erythrocyte D antigen expression. Am J Hematol 2012; **87**: 266-71.

Arrived: 22 October 2014 - Revision accepted: 28 January 2015
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