Determination of Antigen Expression on Cultured Red Blood Cells

Running title: *Determination of cRBC antigen expression*

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Abstract

Background: The *ex vivo* generation of enucleated red blood cells would provide safe and adequate supply of blood for both laboratory use and transfusion purposes. Having continuous supply of reagent red cells to be used in an immunohematology reference laboratory would help with resolution of complex antibody identification cases. Therefore, this study aims to determine if cultured red blood cells (cRBCs) express antigens that are present on native red blood cells (nRBCs).

Methods: CD34+ donor cells were cultured in a serum-free 21 day *ex vivo* culture. Native and cultured red blood cells from the same donor were tested concurrently and expression of antigens were compared. Cells from three donors, identified here as CS495, CS509, and CS510, were utilized to further investigate quantitative antigen expression by titer analyses.

Results: All antigens tested produced same reactions between nRBCs and cRBCs except for Fy^b (donor CS495), and Fy^b and Jk^a (donor CS509). Cultured RBCs tested positive for these antigens, whereas the native RBCs tested negative. Titer studies showed no significant quantitative difference in antigen expression between cRBCs and nRBCs.

Conclusion: Our findings suggest antigen detection on cultured red cells is equivalent to native red cells for majority of antigens; however, discrepancies were noted for Fy^b and Jk^a antigens. These preliminary findings suggest that detailed analyses of antigenicity be considered as methods for *ex vivo* generation of red blood cells advance toward clinical application.

Keywords:

Cultured red cells, red cell phenotyping, antigen expression

Abbreviations:

HSCs	hematopoietic stem cells
RBCs	red blood cells
nRBCs	native red blood cells
cRBCs	cultured red blood cells
HPA	high prevalence antigen
LPA	low prevalence antigen
EDTA	ethyldiaminetetraacetic acid
IRLs	immunohematology reference laboratories

BACKGROUND

Red blood cell transfusion is a treatment used in today's modern medicine and its safety and adequacy are of utmost importance. Many reasons prompted researchers to explore alternative ways to produce adequate amounts of blood for clinical and transfusion purposes. One is the decrease in blood donations due to deferrals in already small donor base and sporadic shortages related to holidays and extreme weather conditions.[1] Additionally, shortages and difficulties involved in finding and supplying rare red cell units and the ever increasing demand for phenotypically similar blood for chronically transfused patients are further reasons to explore alternatives. Limited availability of reagent red cells with atypical phenotypes is another motive.

Improvements in the field of erythroid biology are being sought for the ultimate goal of erythrocyte engineering for clinical use.[2] Erythropoiesis is a very complex process and involves proliferation and differentiation of stem cells into fully developed red blood cells. The *ex vivo* generation of enucleated red blood cells can create opportunities to provide safe and adequate supply of blood for both laboratory use and transfusion purposes. Maintaining a continuous supply of reagent red cells for use in the immunohematology reference laboratories (IRLs) is a possibility since RBCs exhibiting a negative phenotype for high prevalence antigens (HPA) or a positive phenotype for low prevalence antigens (LPA) are difficult to obtain. The same holds true for finding phenotypically similar red cell units for patients with antibodies to HPA. It would be easier finding units for chronically transfused populations, such as patients with sickle cell disease, or patients with rare blood group type that are in need of phenotypically matched RBCs. Worldwide banking of rare bloods is now considered as the only reliable strategy to provide transfusion facilities with blood samples showing atypical phenotypes.[3]

The purpose of this study is to determine if *ex vivo* generated red blood cells express antigens same as do native red blood cells using column agglutination. Peripheral blood CD34+ hematopoietic stem cells (HSCs) are used to grow the cultured red blood cells (cRBCs). The CD34 is a cell surface marker widely used in identification and isolation of hematopoietic stem cells from various sources such as peripheral blood, cord blood, and bone marrow.[4]

METHODS

Donor Selection

All studies using primary erythroblasts were performed after human subjects review and NIH IRB approval and donor informed consent was obtained.[2] In order to reduce the amount of time needed to complete this project, donors were selected from a group of donors that had CD34+ stem cells collected prior to the start of the project. Complete blood counts fell within the normal range for all donors. Five healthy volunteer donors were selected from ten available donors according to their ABO and other blood group phenotypes. Selection was necessary to assure having at least one donor with a double expression of an antigen and a donor with single expression of an antigen within a blood group system. For example, the goal was to have at least one Fy(a+b-), one Fy(a-b+), and one Fy(a+b+) cells when the Duffy blood group system was taken into consideration. This selection process was followed when possible, but based on antigen prevalence, donors with E+e- or K+k- phenotypes could not be used in this study.

Collection and Storage of Samples

Peripheral whole blood samples from each donor were collected into ethyldiaminetetraacetic acid (EDTA) tubes and delivered to the Transfusion Services Laboratory. Samples were kept at 1-6^{o}C until they were droplet-frozen as previously described.[5] Cryopreserved cells were maintained in liquid nitrogen until testing was performed.

Preparation of Cultured Erythrocytes

CD34+ cells were cultured in a serum-free 21 day ex vivo culture as described previously.[6] Culturing was structured in three phases to optimize progenitor (phase 1), erythroblast (phase 2) expansion, and terminal maturation (phase 3).[7] On day 21, the cultured erythrocytes were enriched using Ficoll-Paque Premium (GE Healthcare Bio Sciences,

Piscataway, NJ) according to manufacturer's protocol. After separation, the pelleted erythroid cells were collected and washed twice in phosphate buffered saline, and resuspended in MTS Diluent 2+ (Ortho Clinical Diagnostics, Raritan, NJ) at $2.0x10^6$ cells/50µL for antigen expression analysis.

RBC Antigen Expression

A total of fifteen red cell antigens (A, B, D, C, E, c, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, S, s) belonging to six blood group systems were tested by column agglutination using MTS Anti-IgG Card, ID-MTS Gel System (Ortho, Raritan, NJ). Testing for group B and K antigens served as a negative control for the respective blood group systems. Table 1 shows antisera used in determining red blood cell antigen expression obtained from the following sources: Alba Bioscience (Edinburgh, UK), Bio-Rad (Dreiech, Germany), Immucor (Norcross, GA), and Ortho Clinical Diagnostics (Raritan, NJ). Antisera quality control was performed with reagent red cells before donor testing (data not shown). Reagent red cells were washed and diluted with the same diluent used for donor red cell suspension before performing quality control in order to achieve reproducibility with donor testing. The procedure was modified from manufacturers' directions to achieve consistency in testing. Native and cultured RBCs were diluted to 0.8% cell suspension. Testing was performed by adding 50µL of the 0.8% red cell suspension and 25µL of the antisera to appropriate test column. MTS Anti-IgG Cards were incubated at 37±2°C for 15 minutes. After incubation, prepared cards were centrifuged for 10 minutes in the MTS Centrifuge at the preset conditions established by the manufacturer. The intensity of reactions was graded visually using the MTS gel grading chart (Figure 1).

Titer Study

Titers were performed for Fy^a and Jk^a antigens for donor CS495, group A antigen for donor CS509, e and Fy^a antigens for donor CS510 using column agglutination. Adequate numbers of tubes were labeled with antisera information and dilution identification (1, 2, 4, 8, 16…...512). Reagent antisera (anti-A, anti-e, anti- Fy^a, anti-Jk^a) were serially diluted by adding 200µL saline into all tubes except the first, and 200µL of antisera into the first and second tubes. The contents of tube number two was mixed and 200µL was transferred into the third tube. Dilutions were carried out in this fashion for the remaining tubes using a new pipette tip at each consecutive step to prevent cross contamination. Testing was performed by adding 50µL of 0.8% donor red cell suspension and 25µL of the appropriate diluted-antisera to appropriate test column. Anti-IgG cards were incubated at 37±2^oC for 15 minutes. After incubation, cards were centrifuged for 10 minutes in the MTS Centrifuge at the preset conditions. The cards were read and graded visually using the MTS gel grading chart.

RESULTS

A total of five donors were chosen for this study. Three group O donors (CS519, CS522, and CS510) and two group A donors (CS495 and CS509) were selected and their phenotyping results were retrieved from donors' electronic medical record (Table 2). Cryopreserved native red cells were thawed[5] after the receipt of the cRBCs and prepared into a 0.8% red cell suspension using MTS Diluent 2+ (Ortho Clinical Diagnostics, Raritan, NJ). Cultured and native cells were tested concurrently to prevent variability in testing and results.

Initially, antisera from Ortho Clinical Diagnostics (Raritan, NJ) were used to test for Kidd blood group antigens (Table 1). This produced mixed-field reactivity for both quality control and donor testing. Repeat testing with antisera from Immucor (Norcross, GA) resulted in true positive or true negative reactivity, resolving the mixed-field problem.

Red Cell Phenotyping

Phenotyping on nRBCs and cRBCs were performed using column agglutination instead of the tube method because of the small sample size. Cultured RBCs and native RBCs from the same donor were tested concurrently, and the expression of antigens was compared. Testing was carried out in duplicate for each donor in order to achieve reproducibility and accuracy in the results.

Table 3 shows the summary of phenotyping results for the 5 donors. Almost all of the antigens tested produced same reaction between cRBCs and nRBCs. False positive results were detected for the Fy^b and Jk^a antigens. Donor CS495's native cells phenotyped as Fy(a+b-) but the cultured cells phenotyped as Fy(a+b+). Donor CS509 had two antigens giving different results. Native cells for this donor typed as Fy(a+b-) and Jk(a-b+) but the cRBCs phenotyped as

Fy(a+b+) and Jk(a+b+) respectively. Donors' historical phenotypes were in agreement with nRBC testing results.

Titer Study

This study was done to see if there was any significant quantitative difference in antigen expression between nRBCs and cRBCs. Titer studies were performed on sample remaining after phenotypes were determined. Only one or two antigens for each donor could be tested due to small sample size. Column agglutination was used to maximize sample utilization and consistency. Three of five donors' samples were tested and no significant difference observed between nRBC and cRBC (Table 4). One minor difference was between donors CS495 and CS510. The Fy^a titer for donor CS510 was higher on cRBCs than nRBCs whereas it was higher on nRBCs than cRBCs for donor CS495.

DISCUSSIONS

Despite the significant progress in red cell engineering, generating sufficient amounts of RBCs is still the biggest obstacle in turning *ex vivo* generated RBC transfusion into a reality. A variety of studies are being performed using the cultured cells to make advances towards the eventual goal of clinical transfusion. However, it is important to identify intermediate clinical goals for these *ex vivo* generated cells achievable with current technologies.[7] Current knowledge can be used in producing enough reagent red cells for reference laboratory testing. Thorough immunophenotyping of cultured red blood cells is essential for this purpose.

This study was done to determine if cRBCs expressed blood group antigens in the same way as do nRBCs. Expression of human blood group antigens during erythropoiesis in a cell culture system has been previously studied by flow cytometry analyses.[8, 9] Douay and coworkers have studied RBC antigen expression as part of a larger study to prove the principle of transfusion of *in vitro* generated red blood cells.[10]

To the best of our knowledge, the current study was the first to perform quantitative analyses of antigen expression on cRBCs using titer studies by column agglutination. There were no significant quantitative difference in antigen expression between nRBCs and cRBCs for the antigens with consistent results. In our study, the donor size and the number of antigens tested were small. The method used was not in accordance with the manufacturers' insert yet we were able to detect red blood cell antigens on cultured cells that were present in native cells for most antigens tested. False positive results were detected for Fy^b and Jk^a antigens according to comparison of cRBCs and nRBCs for donors CS495 and CS509. Additional studies are needed to assess whether the genetic program of HSCs, culture constituents, assay design, antisera used

in testing, or any combination of these factors affect evaluation of the blood group antigens on cultured red cells.

The possible interference of Ficoll-Paque was investigated. Cell separation in this media takes place by sedimentation of erythrocytes aggregated by Ficoll PM400/sodium diatrizoate rather than cell density separation alone.[11] Donors' native red blood cells were separated by following manufacturer's instructions and tested by column agglutination. Native cells did not produce a positive result.

Assay design can be evaluated by testing cRBCs under the same conditions in tubes instead of column agglutination. Antisera should also be evaluated utilizing reagents from a different source because antisera components other than the antibody such as preservative and/or antibiotic might have caused nonspecific aggregation of the cultured cells. In addition, no molecular study was done on cultured red cells because donors had not consented for molecular testing.

Overall, these results suggest that immunophenotyping of cultured red cells produce patterns that are nearly identical to those obtained using native cells from the donors. These preliminary findings suggest further investigation is needed to determine the molecular and cellular basis of antigen expression and detection using cultured red blood cells.

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Table 1. Antisera Used*

Antisera Specificity	Antiserum			
	Polyclonal/ Monoclonal	Immunoglobulin Class	Cell Line (clone)	Source
Anti-A	Monoclonal	IgM	LA2	Alba
Anti-B	Monoclonal	IgM	LB3	Alba
Anti-D	Monoclonal	IgM & IgG	BS232, BS221, H41, 11B7	Bio-Rad
Anti-C	Monoclonal	IgM	MS24	Ortho
Anti-E	Monoclonal	IgM	DEM1	Alba
Anti-c	Monoclonal	IgM	H48	Alba
Anti-e	Monoclonal	IgM	MS16	Ortho
Anti-K	Monoclonal	IgM	MS56	Immucor
Anti-k	Polyclonal	IgG	human serum	Immucor
Anti-Fy^a	Polyclonal	IgG	human serum	Ortho
Anti-Fy^b	Polyclonal	IgG	human serum	Ortho
Anti-Jk^a	Monoclonal	IgM	MS15	Ortho
Anti-Jk^b	Monoclonal	IgM	MS8	Ortho
Anti-Jk^a	Polyclonal	IgG	human serum	Immucor
Anti-Jk^b	Polyclonal	IgG	human serum	Immucor
Anti-S	Polyclonal	IgG	human serum	Immucor
Anti-s	Monoclonal	IgG	P3YAN3	Bio-Rad

* Antisera information taken from manufacturers' insert.

Table 2. Donor Phenotype*

	Blood Group Systems												
	ABO	Rh					Kell	Duffy		Kidd		MNS	
DONORS		D	C	E	c	e	K	Fy^a	Fy^b	Jk^a	Jk^b	S	s
CS509	A	+	+	-	-	+	-	+	-	-	+	+	-
CS505	A	+	+	-	-	+	-	+	-	+	-	+	+
CS419	A	+	+	-	+	+	-	-	+	+	-	-	+
CS488	A	+	-	+	+	+	-	+	-	+	-	+	+
CS495	A	+	+	-	+	+	-	+	-	+	-	-	+
CS494	O	+	+	-	+	+	-	+	+	+	-	+	+
CS519	O	+	+	-	+	+	-	-	+	+	+	-	+
CS522	O	+	-	+	+	+	-	-	+	+	+	+	+
CS501	O	+	+	-	+	+	-	+	+	+	+	-	+
CS510	O	+	-	-	+	+	-	+	+	+	-	+	+

* Donor phenotyping results retrieved from electronic medical record.

Table 3. Concurrent testing results for cultured and native red cells

	DONORS									
	CS495		CS509*		CS510*		CS519*		CS522*	
Antigens	nRBCs†	cRBCs‡	nRBCs†	cRBCs‡	nRBCs†	cRBCs‡	nRBCs†	cRBCs‡	nRBCs†	cRBCs‡
A	+	+	+	+	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-
D	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	-	-	+	+	-	-
E	-	-	-	-	-	-	-	-	+	+
c	+	+	-	-	+	+	+	+	+	+
e	+	+	+	+	+	+	+	+	+	+
K	-	-	-	-	-	-	-	-	-	-
k	+	+	+	+	+	+	NT	NT	NT	NT
Fy^a	+	+	+	+	+	+	-	-	-	-
Fy^b	-	+	-	+	+	+	+	+	+	+
Jk^a	+	+	-	+	+	+	+	+	+	+
Jk^b	-	-	+	+	-	-	+	+	+	+
S	-	-	+	+	+	+	-	-	+	+
s	+	+	-	-	+	+	+	+	+	+

* These donors' cells were cultured and tested twice.

† nRBCs = native red blood cells

‡ cRBCs = cultured red blood cells

TABLE 4. Titer Study

	DONORS					
	CS495		CS509		CS510	
Antigens	nRBCs*	cRBCs†	nRBCs*	cRBCs†	nRBCs*	cRBCs†
A	NT	NT	1024	512	NT	NT
e	NT	NT	NT	NT	512	1024
Fy^a	16	8	NT	NT	32	64
Jk^a	64	64	NT	NT	NT	NT

* nRBCs = native red blood cells

† cRBCs = cultured red blood cells

Figure 1. Reaction Grading Chart