Published in final edited form as:

Transfus Med. 2014 April ; 24(2): 114–116. doi:10.1111/tme.12109.

Membrane Attack Complex Generation Increases as a Function of Time in Stored Blood

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SUMMARY

Objective—To determine if the complement system, a potent mediator of inflammation, contributes to hemolysis during RBC storage.

Background—Red blood cells in storage undergo structural and biochemical changes that may result in adverse patient outcomes post-transfusion. Complement activation on leukodepletion and during storage may contribute to the red blood cell storage lesion.

Methods/Materials—We performed a cross-sectional analysis of aliquots of leukoreduced RBC units, stored for one to six weeks, for the levels of C3a, C5a, Bb, iC3b, C4d and C5b-9 (MAC) by ELISA.

Results—We observed that only MAC levels significantly increased in RBC units as a function of storage time. We also observed that the level of C5b-9 bound to RBCs increased as a function of storage time.

Conclusion—Membrane attack complex levels increased over storage time, suggesting that MAC is the primary complement-mediated contributor to changes in stored RBCs. Inhibition of the terminal complement pathway may stabilize RBC functionality and extend shelf life.

Keywords

complement; membrane attack complex; extrinsic protease pathway

INTRODUCTION

It is well established that structural and biochemical changes occur during the storage of red blood cells (RBCs) for transfusion (Weinberg *et al.*, 2011). Changes in older RBCs potentiate adverse patient outcomes post-transfusion, including increased susceptibility to

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

infection, post-surgical complications and death (Weinberh *et al.*, 2011; Gauvin *et al.*, 2010). Although the pathologic relevance of the storage lesion has been questioned (Middelburg *et al.*, 2013), adverse outcomes are common in trauma patients (Weinberg *et al.*, 2011). In this clinical setting, the so-called RBC storage lesion may deliver a “second injury” in which host inflammatory mechanisms contribute to storage lesion toxicity (Kim-Shapiro *et al.*, 2011).

Complement is a potent inflammatory mediator functioning to protect the host against infection, in part, by lysing pathogens *via* the membrane attack complex (MAC) (Esser, 1994). Complement is activated during leuko-reduction of whole blood, generating activation fragments which may contribute to storage lesion toxicity (Seghatchian, 2003). We examined for changes in the levels of six complement activation proteins in leukoreduced RBC units and found that only MAC levels increased over time, suggesting that MAC is the primary complement-mediated contributor to changes in stored RBCs.

MATERIALS AND METHODS

Blood samples

Aliquots were aseptically obtained from leukoreduced units ($n=70$) in additive solution (types, O, A and B) prior to transfusion at the University of Alabama at Birmingham or the University of Tennessee Health Science Center. *Each unit was sampled only one time prior to transfusion.* Study approval was obtained from the institutional review boards at both facilities.

Complement ELISAs

Supernatants were assessed for levels of C3a, C5a, Bb, iC3b, C4d and C5b-9 (MAC) by ELISA (Quidel, Corp., San Diego).

Flow Cytometry

RBCs stored for two to four weeks post-donation were flow cytometric analysis using a monoclonal antibody to human C5b-9 neoantigen (Quidel Corp. San Diego). Stained cells were run on a FACSCalibur and data was analyzed with CellQuest software (BD biosciences).

Statistical analysis

Statistical analysis was performed using Prism 5 (Graphpad Software, San Diego, CA). A p value of less than 0.05 was considered significant.

RESULTS

Levels of C5a, C3a, iC3b and Bb did not *significantly* increase in RBC units stored from one to six weeks at 2–6° C (Fig. 1A–D, Table 1). C4d levels correlated negatively with storage time, but not significantly (Pearson’s correlation= -0.24 , $p=0.09$) (Fig. 1E). In contrast, we observed a significant, time-dependent increase in C5b-9 levels (Pearson’s correlation= 0.15 , $p=0.001$) (Fig. 1F). In addition to changes in soluble C5b-9 levels, we also observed a

significant increase in C5b-9 bound to RBCs as a function of storage time (Fig. 2). Aliquots of RBCs stored for six weeks had significantly higher levels of membrane-bound C5b-9 compared to samples stored for two to three weeks post-donation.

DISCUSSION

Transfusion of banked RBCs represents a highly atypical setting with respect to complement biology and function. Unlike circulating whole blood, complement in stored RBCs is at 2–6°C and diluted in a non-physiological buffering system which changes over time with respect to pH and ionic strength (Gevi *et al.*, 2012). Complement can however, be activated in the cold, indicating that low temperature alone does not prevent activation (Hyllner *et al.*, 1997). Unexpectedly, our data showed a permissive activation of the terminal complement pathway despite inhibition of complement by citrate in additive solutions. Although complement could lyse RBCs under conditions of low antibody concentration as found in units (Götze, 1970), our data suggest mechanisms independent of the traditional pathways, are utilized to generate MAC in stored RBC units. It has been known for several decades that enzymes of the coagulation/kinin systems activate complement (Markiewski *et al.*, 2007). C3 and C5 are cleaved by plasmin, thrombin, kallikrein or activated factors IX–XI through this “extrinsic protease pathway”. These enzymes are also active under conditions used to store blood (Blombeck *et al.*, 1984). Our data suggest that C5 is a primary target of this pathway, leading to damage or lysis of RBCs through MAC formation. In support of this mechanism, we observed a correspondingly higher level of MAC deposition on RBCs as a function of storage time. These studies suggest that MAC formation is an important step in hemolysis during storage and suggest that strategies to inhibit MAC formation may extend RBC storage time.

Acknowledgments

This work was supported in part by NIH grant HL095468 from the National Heart, Lung and Blood Institute to RPP, JAW and SRB. All authors contributed to experimental design, analysis of data and drafting of the manuscript. X. H. and T.N.R. contributed extensively to acquisition of data.

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Figure 1.

Levels of soluble C5b-9 increase over time in RBC units. Levels of complement proteins versus time of storage in days are shown (n=53–70). The line represents linear regression using best-fit values. A. C5a, B. C3a, C. iC3b, D. Bb, E. C4d, F. C5b-9.

Figure 2.

Percent of RBCs with bound C5b-9 as a function of storage time. RBCs stored for two to four weeks post donation were subjected to flow cytometric analysis using anti-C9 antibody that detects a C9 neoantigen expressed on C5b-9. The data shown are pooled from 3–9 independent samples at each time point. Bound C5b-9 levels were significantly different at weeks 2 and 3 compared to week 6 ($p < 0.05$, students t-test).

Table 1

Pearson's correlation coefficient and 95% confidence intervals for changes in complement levels in RBC units over time.

Complement Component	r ² (p-value)	95% confidence interval
C5a	0.012 (0.28)	−0.11 to 0.38
C3a	0.014 (0.36)	−0.13 to 0.36
iC3b	0.043 (0.11)	−0.047 to 0.44
Bb	0.002 (0.72)	−0.21 to 0.3
C4d	0.06 (0.093)	−0.48 to 0.04
C5b-9	0.15 (0.001)	0.15 to 0.58