R-fact study: risk factors for alloimmunization after red blood cell transfusions : methodology, risk factors and challenges in transfusion medicine research

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CHAPTER 4

EFFECT OF STORAGE OF RED BLOOD CELLS ON ALLOIMMUNIZATION

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ABSTRACT

Background

Red blood cells (RBCs) undergo changes during storage. Various studies have suggested a higher risk of adverse and often multi-factorial clinical outcomes associated with older stored RBCs. Our aim therefore was to examine if storage of transfused RBCs is also associated with the risk of RBC specific alloantibody formation.

Methods

A two-center retrospective case- referent study was performed where case patients and control subjects were sampled from all consecutive patients who had received their first and subsequent red blood transfusions in one of the two centers only. Cases were defined as patients who developed a first RBC alloantibody. Control subjects were patients without detectable RBC alloantibodies, who were matched to the case patients regarding number of RBC transfusions. Binary logistic regression analysis was used to examine the association between storage time of RBC and the occurrence of alloimmunization.

Results

A total of 144 cases and 286 controls were selected for our study, who had received a total 5478 RBC units. Comparing patients receiving units stored shorter than a certain number of days versus older units (with various storage periods up to 4 weeks) did not reveal an association or a trend between alloimmunization risk and storage time categories.

Interpretation

Our findings suggest that storage times of transfused RBCs between one and four weeks do not affect the risk of alloimmunization.

INTRODUCTION

Patients undergoing red blood cell (RBC) transfusions are exposed to non-self, donor red cell antigens, and as a result may develop antibodies to such foreign antigens. The alloimmunization risk is influenced by clinical[1] and genetic predisposition[2], as well as by the number of transfusions[3].

Red blood cells undergo various biochemical and biomechanical changes during storage, known collectively as the storage lesions. Such changes include membrane changes, changes in the vaso- dilatory capacity of micro-vessels due to NO release by free hemoglobin, 2, 3-DPG depletion and release of potassium[4,5]. Residual leucocytes and platelets are present, and the accumulation of released lipids, cytokines and histamine have been reported in suspension solution[6,7]. These lipids and micro-vesicles are biologically-active, with pro-inflammatory and pro-coagulant activity[8]. Currently, the clinical importance of these changes in stored RBCs is not clear, and there is much debate on the topic[8-17].The definition of aged red cells varies in the available literature. Most often a storage time period of 14 days[9-12] is used as a cut-off point to define older versus younger blood but this is arbitrarily based on the depletion of 2,3 DPG in 2 weeks stored red cells[4,13]. Other storage lesion markers might well result in another differentiation of young vs. old blood.

It was demonstrated that storage of RBC transfused in mice results in stronger immunization to one very immunogenic model antigen[14]. A similar phenomenon, however, was not observed for anti-D formation following Rhesus D incompatible transfusions in humans[15]. To clarify this issue for current transfusion practice storage times, we studied if the storage time of transfused RBCs was associated with the risk of alloantibody formation in a previously non-alloimmunized transfusion population.

METHODS

Study design and study population

A case referent study at the Leiden University Medical Center, Leiden and the University Medical Center Utrecht, Utrecht, in the Netherlands was performed. This study is part of an ongoing study on Risk Factor for Alloimmunization after red blood Cell Transfusions (R-FACT). The full protocol of the study has been published previously[16]. The study was approved by the ethics committees of both participating hospitals.

The source population consisted of all not previously transfused, non-alloimmunized patients who had received their first RBC transfusion, including at least one pre- and post-transfusion antibody screening, at the same study center. The study period was January 2005 to December 2010 at Leiden University Medical Center and January 2006 to December 2011 at University Medical Center Utrecht, Utrecht. Cases were all patients in the cohort with a first time ever RBC alloantibody. For each case two controls were sampled and matched to case patients based on the total number of red cell transfusions received. This was done following the "risk-set" sampling strategy from the total transfusion cohort, who were at

risk (i.e., had at least received the same number of RBC units) of becoming a case at the time the case was diagnosed[22].

Children were excluded, because in the participating hospitals children routinely received RBC stored for ≤7 days, as a matter of transfusion policy.

Storage time was categorized in weekly periods up to 4 weeks for the analysis.

Implicated Period

An "implicated period" or a "clinical risk period for alloimmunization" was defined for case and control patients as the period in which a transfusion most likely caused the observed primary alloimmunization. This implicated period was the time (in days) between the last transfusion before a first ever positive alloantibody screen and 30 days earlier[16]. To optimize the likelihood that our cases were primary immunizations, and not secondary booster immunizations, we considered an alloimmune response to need a minimum "lag period" of 7 days between the first finding of the antibody and the last preceding transfusion. As part of a study on clinical risk factors, a similar implicating period was selected for the controls[16]. (details presented in the published R-FACT study protocol[16])

Red Blood Cells and First time formed RBC alloantibody

Routinely transfused red blood cells in the Netherlands are in SAGM, pre-storage leukoreduced, not irradiated. RBC alloantibodies were defined as warm reacting clinically significant antibodies (C, E, c, e, C^w, K, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, Lu^a, Lu^b, M, N, S and s), screened for using a three cell panel including an indirect antiglobulin test (LISS Diamed ID gel system) and subsequently identified by a standard 11 cell panel using the same technique. All patients were routinely screened for alloantibodies before transfusions. If transfusions were given at subsequent days, the maximum interval between screens was 72 hours.

Data acquisition

Transfusion dates and dates of donation of every RBC unit received, dates and results of the antibody investigations, patients' dates of birth, gender and ward of hospitalization were gathered from the hospitals' electronic laboratory information management systems.

Data Analysis

Binary logistic regression analysis was used to examine the association between storage time of RBC and the occurrence of alloimmunization. Analysis was conducted based on various cut-off days for younger and older stored RBC units. Patients receiving on average fresher RBC unites were compared to patients receiving on average older units. In addition, patients who had received only younger (fresher) stored RBC units were compared to patients who received only older RBC units, to exclude any effect of the mixed stored (a combination of young and old blood units) RBC units[17].

All odds ratios (ORs, 95% confidence interval, CI) were corrected for the number of transfusions for which the cases and controls were matched. We presented adjusted ORs,

adjusted for possible determinants that could have confounded the association, independent of their statistical significance in the univariate analysis.

Confounders

We corrected for the number of transfusions in the implicating period (or, clinical risk period) since RBC units stored for a longer period have a tendency to be given out to patients requiring low numbers of units. This is due to the often practiced 'first in, first out' inventory management policies. Patients with indications for massive transfusions usually receive shorter stored RBC units, as a result of "first in, first out" inventory management policies. Additional categorical variables were patient age, sex and clinical ward of transfusion requirement (with acute requirements like surgical wards vs non-acute requirements like hematology wards), hospital and year (2005-2011) of donation of the transfused products. The latter two can be regarded as possible indicators of changes in blood collection, manipulation and different local transfusion strategies, if any.

RESULTS

Population Characteristics

During the study period, 17767 patients received first ever RBC transfusion at our study centers and 156 formed clinically relevant RBC alloantibodies.

The study population initially comprised of 468 patients (156 cases, 312 controls). After excluding 38 children, the remaining 144 cases and 286 controls had received a median of 9 RBC units in the study period and median 4 units in the implicated period. The distribution of age, sex, mean storage time per RBC unit and ward of hospitalization in cases and controls are presented in table 1. The median storage time of transfused RBC units was 16 days.

Table 1. Characteristics of RBC alloimmunization case and control patients

Characteristics	Case patients	Control patients
Number of patients	144	286
Males (n) Male to Female ratio	82 (1.3)	161 (1.3)
Age (mean, standard deviation)	57.5 (18.3)	57.5 (18.2)
Total number of RBC units – Median (Inter quartile range)	1834 9 (11)	3644 9 (10)
Total number of RBC units in implicated period – Median (Inter quartile range)	815 3 (4)	1953 4 (6)
Median storage time in implicated period (in days) – (Inter quartile range)	16 (8)	15 (7)
Patients in wards with acute RBC requirements, n (%)	67 (47)	147 (51)

Storage time and risk of alloimmunization

The majority of case patients (50%) were present in the average storage time category of day 15-21 days, (week 3) and was chosen as the reference group, in order to establish a robust comparison group. Patients receiving on average stored blood less than 2 weeks old; and patients receiving on average stored blood more than 3 weeks old compared to week 3 had adjusted ORs for alloimmunization of 1.05 (95% CI 0.6-1.8) and 0.9 (95% CI 0.7-1.3) respectively. (Table 2)

Table 2. Association between storage times considered as the average storage time per patient, categorized into weeks, and the occurrence of alloimmunization

Reference (3rd week)	Cases (n) 72	Controls (n) 144	Crude OR * (95% CI) 1 (ref)	Adjusted OR ** (95% CI) 1 (ref)
≤2 weeks	43	82	1.08 (0.6-1.8)	1.05 (0.6-1.8)
>3 weeks	29	60	1.003 (0.8-1.3)	0.9 (0.7-1.3)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

Table 3. Association between average storage time of RBC unit per patient and risk of alloimmunization: Comparison of average blood older than 14 days to average blood younger than 14 days and the occurrence of alloimmunization

"X" days vs. <= 14 days	Cases (n) 43	Controls (n) 82	Crude OR *	Adjusted OR **
> **14**	101	204	0.9 (0.6-1.4)	0.9 (0.6-1.5)
> **15**	89	175	1.01 (0.6-1.6)	1.02 (0.6-1.7)
> **16**	80	155	1.04 (0.6-1.7)	1.1 (0.6-1.7)
> **17**	61	136	0.8 (0.5-1.4)	0.8 (0.5-1.4)
> **18**	52	120	0.7 (0.4-1.2)	0.7 (0.4-1.2)
> **19**	46	98	0.8 (0.4-1.4)	0.7 (0.4-1.3)
> **20**	36	80	0.7 (0.4-1.4)	0.7 (0.4-1.4)
> **21**	29	60	0.8 (0.4-1.4)	0.7 (0.4-1.4)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

Next, patients transfused with units with average storage times more than 14 days up to more than 21 days were compared to patients with transfused units with average storage times less than 14 days and observed no differences, with adjusted ORs in the ranges of 0.7-0.9 (95% CI ranges 0.4-1.7) (Table 3)

Lastly, to try and disentangle the effect of mixed blood (fresher and older stored transfused units) and compare only old versus only fresh transfused blood, patients receiving only RBC units stored for less than 14 days were compared with patients receiving RBC units stored only longer than 14 days up to only longer than 21 days and observed no clear association. Only in patients receiving exclusively units stored for more than 21 days as compared to less than 14 days, an adjusted OR of 0.4 (95% CI 0.1-1.01) was found.(Table 4)

Table 4. Association between storage time of RBC units per patient and risk of alloimmunization: Comparison of patients receiving only more than 14 days stored blood to patients receiving only less than 14 days stored blood

"X" days vs. <= 14 days	Cases (n) 25	Controls (n) 51	Crude OR *	Adjusted OR **
> **14**	75	138	0.9 (0.5-1.7)	0.9 (0.5-1.8)
> **15**	62	116	0.9 (0.9-1.8)	0.9 (0.5-1.8)
> **16**	54	100	0.9 (0.5-1.9)	1.01 (0.5-1.9)
> **17**	42	90	0.8 (0.4-1.5)	0.7 (0.4-1.5)
> **18**	37	80	0.7 (0.3-1.4)	0.6 (0.3-1.3)
> **19**	34	69	0.7 (0.3-1.5)	0.6 (0.3-1.4)
> **20**	26	51	0.8 (0.4-1.7)	0.7 (0.3-1.6)
> **21**	21	43	0.6 (0.3-1.4)	0.4 (0.1-1.01)

* Odds ratio adjusted for number of matched transfusions
** Odds ratio adjusted for number of matched transfusions, sex, ward of hospitalization, hospital, patient age and transfusions received in implicated period

DISCUSSION

In our case-referent study in a previously non-transfused, non-immunized population, storage times of RBC units were not associated with the post-transfusion risk of alloimmunization against clinically significant RBC antigens. We did observe an association between storage time and alloimmunization at the >21 days versus ≤14 days comparison cut-off; however, given our study size, this could be a chance finding.

To our knowledge, this is the first study examining the effect of clinically relevant storage times of red blood cells and clinically relevant alloimmunization against non-AB and non rhesus D RBC antigens that patients normally are not matched for.

The association between the receipt of older stored red cells and other adverse clinical outcomes has been studied before[10,11,20,21]. Most of the available literature has suggested adverse effects of the older stored red cells on clinical endpoints such as post-operative infections and complications, multi-organ failure and mortality. There is no agreement, however, on whether these associations are causal[4].

By excluding children from our study, a source of confounding by indication was avoided, since children were routinely transfused (at both the study centers) with blood stored for less than 7 days. In addition, we adjusted for numerous variables in our study that could have had an influence on the storage time of RBC as well as the risk of alloimmunization.

Failure to detect case patients in our study cohort (measurement bias) might be due to the variable follow-up in post transfusion antibody screening. The study design with "risk- set" sampling of our controls from the source cohort, we feel prevents this possible measurement bias from affecting our exposure (storage time of red cells) distribution in the different comparison groups[20]. Furthermore, we cannot unequivocally rule out secondary immunizations. However, by including only pre-transfusion non-alloimmunized patients as well as introducing the 7 day lag period between first antibody detection and the date of the last preceding transfusion to define the implicated transfusion period, we feel to have efficiently minimized booster reactions. A relative limitation of the study was that there were very few patients who received only red cells stored for less than 7 days or only red cells stored for more than 28 days. The effect estimates of these extreme ends of storage time on alloimmunization were therefore not reliable (wide 95% CIs, data not shown). The inability to obtain such extreme end data – although of conceptual value – seems to indicate their small relevance for day to day practice.

Our 95% confidence intervals clearly justify our conclusions for case and control patients who received RBCs within the storage time ranges of 7-28 days. This storage time range is a reflection of the average RBC storage time range with which 95% of patients in our study centers are transfused.

A hypothesis which could be considered for our observed lack of association with the older stored RBC units (as well as fresher RBC units) could be that two different and opposing "heightened" periods of danger may exist – 1) increasing immunogenicity by leukocyte activity in fresher units that decreases with storage, and 2) immunogenicity by accumulation of cytokines, lipids, histamines and micro-vesicles that increases with storage; where these might largely cancel out each other's effect.

So far murine studies have shown that exclusive transfusion of older units of red cells (expressing a model antigen consisting of hen egg lysozyme fused to ovalbumin fused to human Duffy[b] (HOD antigen) resulted in a *stronger* antibody response than fresh units. Moreover, the co-incident transfusion of fresh RBCs dampened the immunogenicity of the stored RBCs[17]. It is worth noting that these findings involved the height of antibody titers

against a very immunogenic antigen causing universal immunization. The non-AB, non-D RBC antigens in humans that are not matched for in transfusion practice, are usually much less immunogenic. RBC alloimmunization for non AB, non rhesus D antigens in humans, as a function of RBC storage, therefore can only be studied in very large observational datasets. With this, we have to accept that the routine assays around these immunizations do not include validated antibody titer determinations. The immunization frequencies in humans, as in our study, therefore cannot be directly compared with results on antibody titers in universally immunized mice. Additionally, alloimmunogenicity in the murine studies did not substantially increase until a point in storage that can be considered to be equivalent to 42 days of human RBC storage.

A potential important modulator for immunization that studies of this type neither are able to study involve donor RBC and patient factors that determine RBC survival in the patient's circulation. This post transfusion survival[23] as 'biological age' of a given unit might likely be more important for alloimmunization than the "chronological (storage) age". Currently, as RBC survival studies are not routinely done in clinical studies, this issue cannot be assessed with normal laboratory practice.

In conclusion, our patient population, study design and storage time ranges showed that so far, there is no evidence that pre-transfusion storage time (within the ranges of 7-28 days) of red blood cells modulates the risk of alloimmunization. Some indication of a protective effect of older RBC (older than >21 days) was seen but given the study size, this may well be a chance association.

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