The accumulation of lipids and proteins during red blood cell storage: the roles of leucoreduction and experimental filtration

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Abstract

Pre-storage leucoreduction has been universally adopted in most developed countries in Asia, Europe and the Americas. It decreases febrile transfusion reactions, alloimmunisation to HLA antigens, cytomegalovirus exposure, the accumulation of a number of pro-inflammatory mediators in the supernatant, including the accumulation of platelet- and leucocyte-derived proteins and metabolites during routine storage. This review will highlight the lipids and proteins, biological response modifiers (BRMs) that accumulate, their clinical effects in transfused hosts, and methods of mitigation.

Keywords: red blood cells, transfusion, transfusion-related acute lung injury (TRALI), leucoreduction, neutrophils.

Introduction

Transfusion of red blood cells (RBCs) has saved numerous lives, far outnumbering any adverse events induced by their infusion. RBC transfusions allow for lengthy and complicated surgeries, survival from life-threatening injuries in both military and civilian settings, organ, bone marrow, and stem cell transplantation, treatment of malignancies with myelotoxic chemotherapy, survival from haemorrhagic diatheses, and haematologic disorders in which RBC production is significantly decreased or destroyed. While the benefits of transfusions far outweigh the risks of a reaction, these reactions still occur, and therefore efforts have been made to improve haemotherapy in order to further decrease clinical morbidity and mortality.

Pre-storage leucoreduction of RBCs (LR-RBCs) by buffy coat depletion, simple filtration, or a combination of the two removes leucocytes and platelets from the RBC units. Buffy coat depletion causes a one log depletion of both leucocytes and platelets while filtration decreases leucocytes by more than 3 logs and platelets by 2 logs[1]. Universal pre-storage leucoreduction significantly decreases febrile non-haemolytic transfusion reactions and decreases exposure to HLA antigens, HLA alloimmunisation, and decreases the accumulation of platelet and leucocyte derived pro-inflammatory mediators, biological response modifiers (BRMs), including: interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), soluble CD40 ligand (sCD40L), and lysophosphatidylcholines (lyso-PCs)[2-8]. Despite these decreases, pro-inflammatory mediators still accumulate during routine storage of LR-RBCs, a number of which have been implicated in the pathogenesis of transfusion-related acute lung injury (TRALI) and post-injury multiple organ failure (MOF), which includes acute lung injury (ALI)[5,9]. This review will detail the mediators/BRMs in question, their clinical effects and possible mitigation, as well as proposing novel strategies to inhibit their production during routine storage.

Transfusion-related acute lung injury

TRALI, which is a rare, adverse event, has been linked to the infusion of bioactive lipids which accumulate during the routine storage of RBCs, and are released into and accumulate in the supernatant of the RBC units[5,10-12]. In unmodified RBCs, there are two classes of lipids, based upon their retention time *via* normal phase with further characterisation by reverse phase high pressure liquid chromatography (HPLC) and identification by mass spectrometry: a mixture of lyso-PCs and non-polar lipids consisting of arachidonic acid, 5-hydroxyeicosotetraenoic acid (HETE), 12-HETE and 15-HETE. These data have been reported by a number of other groups[5,8,13-20]. These lipids were increased in patients at the time TRALI was recognised, and both the supernatants and the lipids from stored RBCs, both day (d)28 and d42 of storage, induced TRALI as the second event in a 2-event animal model[5,12,21,22]. Pre-storage leucoreduction by filtration, specifically the Haemonetics BPF4 filter, removes two logs of platelets and the lyso-PCs from LR-RBC units[1,5,12]. The neutral lipids are not affected and may still serve as the second event in a 2-event animal model of TRALI[5,12,22]. In addition, this removal of platelets also decreases the accumulation of sCD40L, a reported co-factor in TRALI, which has the capacity to alter PMN physiology, e.g. prime the PMNs through the CD40 receptor on the cellular membrane[3]. Importantly, animal models are employed to mimic human disease and to give relevance to suspected mediators; however, just because each and

every rodent experiences TRALI, for example, does not mean that each and every human will also manifest this adverse event[23].

The accumulation of bioactive lipids has been questioned; however, these studies measured lyso-PCs in LR-RBCs, by both buffy coat removal and filtration[24]. Pre-storage leucoreduction nullifies the accumulation of these lipids because of effective platelet removal (approximately 2 logs). Moreover, flow-based measurement of oxidase activity is a qualitative test, and because of time constraints, it is not amenable to quantification since the actual assays are not done simultaneously like the 96-well plate assays that measure superoxide dismutase-inhibitable reduction of cytochrome c[1,24]. Lastly, in a prospective clinical study of TRALI, bioactive lipids (lyso-PCs) were risk factors for TRALI in the univariate but not the multivariate analyses[25]. In addition, the bioactivity measurement on PMNs, increased surface expression of CD11b/CD66, did not demonstrate significant pro-inflammatory activity. However, the details of these assays are important because: 1) the bioactive lipids present: AA and 5-, 12-, and 15-HETEs affect the surface expression of CD11b in five minutes and with longer incubations the surface expression disappears; and 2) if fixed with paraformaldhyde prior to incubation with the antibodies to CD11b/CD66, the antigens are changed for CD11b such that the increased surface expression may be diminished by more than 30%[25]. As stated above, lyso-PCs and sCD40L do not accumulate in LR-RBCs due to platelet removal by the filter and the analyses looked at lipids that should not be present in the RBCs but would be in the platelet concentrates[3,5,25].

TRALI mitigation and experimental filtration

Transfusion-related acute lung injury mitigation has centred on the male-only plasma donors to obviate female plasma which may contain antibodies to human lymphocyte antigens (HLA) or human neutrophil antigens (HNA) due to pregnancy. These efforts have significantly decreased TRALI secondary to plasma transfusion but have not eliminated it[26-28]. Nevertheless, there are few formal mitigation strategies for RBC transfusions and reported clinical series have shown that 20% of TRALI follows RBC transfusions, with this percentage likely to increase because of the decrease in TRALI to plasma[26,27,29]. RBCs contain 5-10 mL of plasma so the relative amount of antibodies to HLA or HNA antigens is relatively sparse compared to plasma or even apheresis platelets, although only 10-20 mL of antibody-containing plasma may elicit TRALI[30,31]. To this end, an experimental filter was developed that removes virtually two logs of IgG. Filtered plasma samples from multiparous females known to have antibodies to HLA or to HNA-3a were deemed negative *via* measurements with Luminex™ beads and flow cytometry at two HLA reference laboratories or for HNA-3a at the Granulocyte Laboratory, Blood Center of Southeastern Wisconsin, USA, employing standard techniques in a blinded fashion[1]. Lastly, these experimental filters also removed neutral lipid priming activity which accumulates during routine storage. (This will be discussed under the proteomics section)[1].

TRALI modelling

The 2-event model of TRALI has been recently criticised because humans given endotoxin (LPS) from *E. coli* followed by stored LR-RBCs or the lipids from LR-RBCs did not manifest TRALI[32,33]. Unfortunately, these studies are marred by a number of factors, most of which appeared in the literature many years ago. In rats, LPS from *E. coli* may not be an effective first event; activation of the pulmonary endothelium did not result in PMN sequestration, which is to be expected because rats are known to live successfully in sewers, which have high levels of *E. coli* and *E. coli* LPS from human waste. Thus, for all rodent experiments, the first event was LPS from *S. enteritides* given *via* an intraperitoneal injection[11,34,35]. This first event caused the animals to become: 1) febrile with rigors and shaking; 2) tachypneic; and 3) despondent, although they respond to pain, with all rats having copious diarrhoea[1,11,22,34-37]. On the cellular level, IP *S. enteritides* LPS in rats causes activation of the pulmonary endothelium and sequestration of PMNs to the capillaries as evidenced by increased pulmonary myeloperoxidase and the lung histology without ALI[1,11,22,37]. The *S. enteritides* LPS concentration administered is 2 mg/kg with 99% animal survival[1,11,22,37]. Although critics of this model have deemed this dose to be supra-physiological, 2 individuals were injected with 2 μg-1 mg of either *E. coli* or *S. enteritides* and both became acutely ill with fevers, hypotension, gastroenteritis, increased respiratory rate, somnolence, and malaise, with one admitted to the intensive care unit with mild ALI and multi-organ dysfunction; both survived[38-40]. Additionally, the treatment of human neurosyphilus was LPS infusion that reached 1 mg intravenous (IV) with the overwhelming majority of the patients surviving[38]. Recent human TRALI models gave *E. coli* LPS IV at a concentration of 2 ng/kg which corresponds to 40 pg/mL of plasma for males and 48 pg/mL of plasma for females and resulted in fever over 38 °C, pulse rates of over 90 beats/min, and mild tachypnea with respiratory rates over 20 breathes/min[32,33]. There was no evidence that any of the human subjects had pulmonary endothelial activation or PMN sequestration in the lung, prerequisites for the 2-event model of TRALI[32,33]. *In*

vitro LPS, whether from *E. coli* or *S. enteritides* did not cause significant activation of human pulmonary microvascular endothelial cells (HMVECs), as measured by increased surface expression of intercellular adhesion molecule-1 (ICAM-1) or chemokine release, until a concentration of 20 ng/mL was reached[9,41,42]. In addition, LPS primes PMNs; however, *E. coli* LPS did not prime fMLF activation of the respiratory burst of human PMNs at concentrations of 2 ng/mL and did induce priming of the oxidase at 20 ng/mL but to a lesser extent compared to *S. enteritides* LPS, which was reported to have an almost identical concentration curve for PMN priming of the fMLF-activated respiratory burst and lyso-PC activation of the oxidase[42]. Unlike intact animals, there is no way to process or excrete the LPS, and the human modelling used concentrations much less than the concentrations needed to cause physiological changes in human cells; thus, clinical TRALI from the human modelling is unlikely because of an insufficient first event[43-45]. Lastly, the administration of LPS (intravenous *vs* intraperitoneal) may also have ramifications for its suitability as the first event of a 2-event model of TRALI in humans.

The proteome of the RBC supernatant

To determine the role of pre-storage leucoreduction on the release of proteins during routine storage, 5 units of red blood cells were drawn; 50% (by weight) were left unmodified and the other 50% was pre-storage leucoreduced by filtration. Both were stored in AS-5[46]. The protein concentration increased 2-3-fold in both the unmodified- and LR-RBCs from day (d)1 to d42 of storage[46]. Leucoreduction decreased the total number of proteins in the supernatant from 401 to 231, and of these, 84 proteins increased (>2-fold increase) with 42 being unique to d42, 30 decreased (<2-fold decrease) with 7 being unique to d1, and 117 remained unchanged[46]. Preliminary data with 3 RBC/LR-RBC units from female donors compared to 3 RBC/LR-RBC units from male donors only demonstrated an increase in pregnancy zone protein, which is increased in the female sex[46]. As expected, the leucocyte and platelet-derived proteins, present in the unmodified RBCs, were not present in the LR-RBC supernatant. However, the glycolytic enzymes were more pronounced in LR-RBC supernatant, including: transaldolase, fructose-bisphosphate aldolase, phosphoglycerate kinase, and α-enolase[46]. Other proteins of interest that increased in the LR-RBC supernatant included: latexin (also known as endogenous carboxypeptidase inhibitor and implicated as a mediator of the haematopoietic stem cell compartment), Prdx1, Prdx2, and Prdx6. These all increased during storage in the LR-RBC supernatant likely due to protease activity. Importantly, Prdx6 contains a phospholipase domain which requires either acidic pH or T-phosphorylation for activity; immunoblotting of the Prdx6 in LR-RBCs showed T-phosphorylation indicating an active enzyme[38,47-50]. There was also significant accumulation of MMP-8 and MMP-9, which display extracellular protease activity, most proteosome subunits, and a drastic decrease in cystatin C[46,51,52]. The presence of an active phospholipase in LR-RBCs may explain the accumulation of AA and 5-, 12-, and 15-HETEs, which have been implicated in TRALI[5]. In addition, these lipids can be used as not only the second event, but also the first event in a 2-event animal model of ALI.

RBC supernatant lipids and proteins and the injured patient

Massive RBC transfusion, more than 6 units in the first 12 hours, was an independent risk factor for the development of post-injury MOF[53-56]. With a more conservative transfusion target, haemoglobin of 7.0 g/dL, the transfusion of fewer RBCs has resulted in less MOF, despite increasing patient age and increased injury severity scores, both risk factors for MOF[57]. In these early studies that controlled for the number of RBC units transfused, older, stored RBCs were implicated in MOF[56]. As stated, MOF has decreased; however, post-injury ALI still plagues more than 12.5% of severely injured patients ISS more than 17[58]. In older LR-RBCs, neutral lipids accumulate, notably AA and 5-, 12-, and 15-HETEs, and pilot data have demonstrated that they induce activation of HMVECs and human liver sinusoidal endothelial cells (LSECs) at concentrations that would be reached by 2, 4 and 6 units of LR-RBCs transfused[5].

The proteome of LR-RBCs and that of the injured patients may provide some insight into the development of trauma-induced coagulopathy (TIC). Recent work on TIC has subdivided trauma patients based on their thrombolytic phenotype: systemic hyperfibrinolysis, physiological fibrinolysis and fibrinolysis shutdown[59,60]. A number of proteins in the LR-RBC supernatants have an affinity for plasminogen, especially α-enolase which is the plasminogen cellular receptor, and may be involved in the prolongation of TIC with respect to fibrinolysis: shutdown, physiological or hyperfibrinolysis[9].

Preliminary data have implicated a role for α-enolase in injured patients at risk for ALI (based on the number of transfusions) who also have evidence of fibrinolysis shutdown. These patients are also prone to organ injury, as well as venous thromboembolism (VTE). *In vitro*, α-enolase significantly increased ICAM-1 surface expression on HMVECs and induced the adherence of PMNs to these activated endothelial cells[9]. This HMVEC activation was inhibited by anti-proteases, required human plasma, and served as the first event in a 2-event

model of PMN cytotoxicity[9]. α-enolase was shown to also co-precipitate with PAR-2 and plasminogen/plasmin in HMVECs and enzymatic activity was not required[9]. Thus, proteins that accumulate during RBC storage as a risk factor for ALI, such as α-enolase, may also elicit previously unrecognised adverse clinical events, both TIC and ALI.

Possible mitigation

Experimental filtration of RBC units, as discussed above, not only removes 2 logs of IgG but it also significantly decreases the priming activity and obviated stored RBCs as the second event in a 2-event animal model of TRALI[1]. In addition, the measured concentrations of AA and 5-HETE were also decreased in the units that underwent experimental filtration *vs* those that were just leucoreduced using the Haemonetics BPF4 filter[1]. As stated previously, active Prdx6 accumulates during RBC storage. When inhibitors of phospholipase activity were added (aristocholic acid and MJ33, a specific inhibitor of the Prdx6 phospholipase), the generation of lipid priming activity was significantly decreased by 25±3% and 26±2%. In addition, when the structure of 5-lipoxygenase activating protein (FLAP) and 5-lipoxygenase were investigated, they demonstrated more than 10% homology with IgG and thus may be removed by the experimental filters. To investigate this removal, immunoblots from pre-filtration supernatants, and supernatants from both the leucoreduced (control) or experimentally filtered units, demonstrated that the FLAP and 5-LO immunoreactivity, present in pre-filtration and in leucoreduced supernatants, was removed by the experimental filters (Figure 1). These data demonstrate that these experimental filters not only remove the immunoglobulins implicated in TRALI, but also the enzymes required to generate the neutral lipids during storage, which have been implicated in both TRALI and post-injury ALI[1].

Conclusions

Pre-storage leucoreduction of RBCs results in fewer febrile transfusion reactions, decreased HLA alloimmunisations, decreased exposure to CMV, and decreased amounts of pro-inflammatory molecules including leucotrienes, lyso-PCs, and sCD40L. It also decreases the release of proteins from contaminating leucocytes and platelets. The non-polar lipids which do accumulate may be obviated by the use of a new leucoreduction filtration system, and possibly by the use of additive solution-3 (AS-3) and other novel storage methods. While transfusions of LR-RBCs has saved countless lives, further work is needed to continue to improve efficacy.

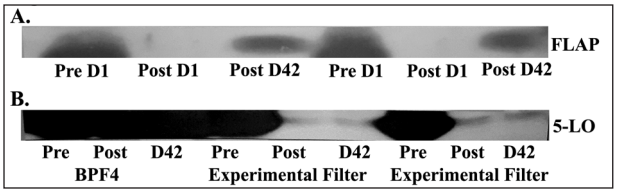

Figure 1 - Pre-storage experimental filtration removes 5-lipoxygenase activating protein (FLAP) and 5-lipoxygenase (5-LO) from red blood cell (RBC) units.
(A) There is significant FLAP immunoreactivity prior to filtration in day 1 (Pre D1) supernatants that is removed by filtration (Post D1) which re-accumulates during routine storage by day 42, the end of storage (Post D42). The supernatants from two different RBC units were used. (B) There is significant reactivity for 5-lipoxygenase (5-LO) both before (Pre) and after (Post) leucoreduction and at the end of storage (D42) with the Haemonetics BPF4 filter. The 5-LO immunoreactivity is present prior to experimental filtration (Pre) that is removed from the supernatant of 2 separate RBC units that does not re-accumulate during routine storage D42. Figure representative of 3 separate experiments which demonstrated similar results.

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