A Reductionistic Approach to Aged Blood

The changes that take place in blood components during storage have long been suspected as a culprit for part of the deleterious clinical effects attributed to blood transfusion. The case against "aged" blood can be made on two fronts: the clinical (often observational) evidence linking the advanced age of blood with adverse outcomes and the laboratory (experimental) evidence showing the changes in biology, chemistry, physics, and rheology as possible mechanisms of action. In this issue of ANESTHESIOLOGY, Vlaar et al.[1] provide direct mechanistic evidence on the effect of storage of blood on lung injury and attempt to pinpoint the element(s) of blood that are adversely affected by storage and responsible for the observed harm using an animal model.

The effective "shelf-life" of donated blood is affected by storage condition and preservative and additive solutions. For years, the US Food and Drug Administration has specified a 42-day expiration date for properly processed erythrocyte units stored in refrigerator, and any erythrocyte units stored for less than this period are deemed suitable for transfusion. However, the list of studies linking extended storage of blood (still within the 42-day permissible period) with unfavorable outcomes (e.g., morbidity and mortality) in various patient populations, including trauma, cardiac surgery, and critically ill patients, has steadily been growing (see Refs. 4–11 in the article of Vlaar et al.[1]).[2–4] Nonetheless, not all studies support this hypothesis,[5,6] and a recent review of 24 studies concluded that the relationship between the age of transfused blood and the patients' outcomes cannot be determined based on available clinical evidence, with the possible exception of massively transfused trauma patients suggestive of a possible dose-dependent effect in addition to the aged blood.[7,8] Available studies are too heterogeneous to allow effective meta-analysis of the results, and the few available controlled trials often suffer from limited sample size

and vaguely defined, overlapping arms.[7,9] A number of large randomized trials have been designed to address these shortcomings. Notably, the Red Cell Storage Duration Study focusing on patients undergoing complex cardiac surgery,* the Age of Blood Evaluation trial in the resuscitation of critically ill patients,† and the Age of Red Blood Cells in Premature Infants Study‡ are currently ongoing. These trials will hopefully provide the definitive clinical evidence on the effect of age of blood on patients' outcomes.

Parallel to the ongoing clinical investigations, preclinical studies have provided invaluable insight into the physiology of the storage effect. Several reversible and irreversible changes that take place in blood during storage, such as decrease of adenosine triposphate, pH, and 2,3-diphosphoglycerate; modification of proteins and lipids; release of potassium, hemoglobin, and other intracellular components, and membrane and cytoskeletal alteration (collectively known as storage lesion), are well documented, and many others are being characterized. However, the significance of these changes in terms of causing clinically evident harm to the recipients of stored blood is uncertain, and further research is needed.[10,11] To this end, the study by Vlaar et al.[1] provides an interesting experimental insight into the link between blood storage and a relatively common and serious complication of blood: transfusion-related acute lung injury (TRALI).

TRALI is the leading cause of transfusion-related death and is characterized by acute-onset lung injury within 6 h of receiving any allogeneic blood components, particularly plasma or plasma-containing products.[12–14] Initial observations indicated that the pathogenesis of TRALI was antibody-mediated, with donor's antibodies against the recipient's human leukocyte antigen class I and human neutrophil antigens or human leukocyte antigen class II antigens on monocytes, resulting in activation and sequestration of granulocytes in lung and endothelial damage. However, subsequent studies showed that these antibodies are neither necessary nor sufficient for TRALI to happen because in some cases, TRALI develops despite the absence of any detectable related antibodies and, moreover, many recipients of blood components containing high titers

* Red Cell Storage Duration Study (RECESS). Available at: http://clinicaltrials.gov/ct2/show/NCT00991341. Accessed March 9, 2010. The study was temporarily suspended for protocol revision following the accessed date mentioned here.

† Age of BLood Evaluation (ABLE) trial in the resuscitation of critically ill patients: A multicentre randomised controlled superiority clinical trial. Available at: http://www.controlled-trials.com/ISRCTN44878718/. Accessed September 9, 2008.

‡ Age of Red Blood Cells in Premature Infants Study (ARIPI). Available at: http://www.clinicaltrials.gov/ct2/show/NCT00326924. Accessed September 30, 2009.

of antibodies linked to TRALI do not develop TRALI. These observations gave rise to another putative mechanism for TRALI, known as the "two-hit" or "two-event" hypothesis. As the name suggests, in this model, TRALI results from two events: one event is patient-derived and results in endothelial activation and granulocyte priming (*e.g.*, surgery or sepsis); the other event is delivered by transfused blood and results in activation of granulocytes (*e.g.*, presence of donor's antibodies against the recipient's leukocytes or presence of proinflammatory agents as a result of extended storage of blood).[13] Thus, this model may directly link TRALI to age of stored blood.

In the study by Vlaar *et al.*,[1] investigators procured blood from Sprague–Dawley rats and processed it in a manner similar to donated human blood to produce packed erythrocytes (albeit without leukoreduction). They transfused the product freshly or after 14-day storage at 4°C to healthy and lipopolysaccharide-primed animals of the same breed at 10% of circulating volume (roughly equivalent of a one-unit packed erythrocyte transfusion in human). Other variants, namely washed stored erythrocytes and the supernatant of the stored erythrocytes, were also prepared and infused. The animals were killed 6 h after transfusion and studied for the signs of lung injury.

Biochemical changes consistent with "storage lesion" were detected in the packed erythrocytes stored for 14 days, with the exception of lysophosphatidylcholine accumulation, which is reported elsewhere as having a role in activating granulocytes[15] but was not detected in this study. Transfusion of aged blood to healthy animals resulted in histologically verified lung injury, whereas the same was not observed using fresh packed erythrocytes. However, transfusion of aged erythrocytes to lipopolysaccharide-primed animals did not further augment the lipopolysaccharide-caused lung damage but only worsened pulmonary coagulopathy and was associated with a nonsignificant increase in pulmonary cytokine and chemokine levels. Interestingly, transfusion of either washed aged erythrocytes or supernatant of aged erythrocytes to healthy animals had similar results (increased pulmonary cytokine or chemokine levels), and washing of the erythrocytes did not prevent lung inflammation in healthy rats. Unfortunately, no histologic scoring for this experiment was reported. On the contrary, only the supernatant of aged erythrocytes was able to increase the pulmonary cytokine or chemokine levels and coagulopathy (again, with no histologic scoring available) in the lipopolysaccharide-primed animals. Interestingly, it seems that not only did the washed aged erythrocytes not increase the cytokine or chemokine levels but also they had a protective effect in lipopolysaccharide-primed animals, because the figures indicate that levels of interleukin-6 and cytokine-induced neutrophil chemoattraetaut-3 in lung homogenates of lipopolysaccharide-treated animals that received washed aged erythrocytes were approximately half of those in lipopolysaccharide-treated control animals that received saline or fresh erythrocytes. This could be the result of noncomparable ex-

perimental conditions, but this observation merits further explanation and exploration.

This study presents an interesting *in vivo* model to study the relation between age of blood and lung injury and possibly other end points. The authors closely reproduced conditions resembling human transfusions by meticulous processing of procured blood and performing within-species transfusions. Nonetheless, one key difference with human allogeneic transfusion is the fact that this is a syngeneic transfusion model, meaning that there is minimal (if any) immunogenic difference between the blood donors and recipients. This is not the case with allogeneic transfusions in humans, in which antigenic differences between the donors and the recipients exist and immunologic consequences are well established. Elimination of the immunogenicity of transfusion in this model can be viewed as a vice and a virtue: it makes the model simpler and the results easier to interpret by avoiding the confounding effect of immunologic reactions but reduces its clinical relevancy at the same time. Genetic differences between the Sprague–Dawley rats obtained from difference breeders have been documented,[16] and this may provide an option for future *in vivo* allogeneic transfusion models, particularly for multiple-exposure experiments.

The results of this study are very interesting, but certain issues interfere with interpretation of the results and conclusions. Of note, better controls could have been used. Head-to-head comparison of washed aged erythrocytes and the supernatant of aged erythrocytes is intriguing, but these two components are very different, and other controls (*e.g.*, washed fresh erythrocytes and the supernatant of fresh erythrocytes) could have improved the comparisons. Indeed, this approach is likely to miss possible properties emerging from the interaction of aged erythrocytes and supernatant, and other alternatives, such as reconstituted blood products with combinations of fresh and old erythrocytes and supernatant, may be worth consideration. Furthermore, having some measures of actual lung injury (*e.g.*, histologic scoring) from the washed erythrocyte *versus* supernatant experiments could have been helpful. The possible effect of prestorage leukoreduction is another important issue, but technical difficulties may pose challenges in this animal model. The reported data suggest that lipopolysaccharide induced widespread lung injury to the extent that aged erythrocytes' effect was overwhelmed. Using lower lipopolysaccharide dosage to limit the damage could have made the experiments more sensitive to the potential effects of blood transfusion. The limited sample size in the experiments and the increased likelihood of type II error should be remembered in interpreting the results. Finally, from a clinical prospective, histologic and biochemical changes are important, especially if accompanied by functional changes (loss of lung function and development of hypoxia in this context). Hence, the measures of pulmonary function and hypoxia should be the ultimate end points in these types of experiments.

The study by Vlaar *et al.*[1] provides some support for the two-hit model of lung injury and the role of age of blood, but

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it remains far from definitive evidence. Each single hit in this study (pretreatment with lipopolysaccharide and transfusion of aged blood) seems to be capable of causing lung injury on its own, and the synergistic effect of the two seems to be limited to a more extensive pulmonary and systemic coagulopathy. Lack of detection of lysophosphatidylcholine accumulation after blood storage is another mystery, given the suggested central role of lipids as the granulocyte-activating agents in the two-hit model.

Nonetheless, if proven to be the case, the two-hit hypothesis would have important clinical implications. First, it directly links the leading cause of transfusion-related mortality, TRALI, to age of blood and is likely to add to the concerns surrounding transfusion of aged blood. Second, it helps in identifying patients at risk of developing TRALI based on a hit that they have already acquired (*e.g.*, sepsis). Finally, it can aid clinicians in preventing TRALI in susceptible patients by preventing the second hit from occurring through, for instance, prioritizing these patients to receive newer available units of blood if they needed transfusion.

With the results of the ongoing trials on the age of blood expected within a year or two, the word "prioritize" may be the key here. What if the age of blood is demonstrated beyond doubt to be a risk factor for adverse outcomes, and a new significantly shorter expiration date is suddenly mandated for each unit of blood? The moment the lines are redrawn, we will find ourselves with a shrunken blood supply in the face of unchanged demand. Will the current narrow margins between the blood donations and the blood transfusions withstand this newly imposed demand?[17] The answer may lie in identifying the patients who benefit most from transfusions and especially those who are likely to benefit most from the newer units of blood.

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