Prestorage leucocyte reduction of red cell components prevents release of bactericidal permeability increasing protein and defensins

E. J. Fransen,1 E. Rombout-Sestrienkova,2 E. C. M. van Pampus,3 W. A. Buurman,4 C. P. M. Reutelingsperger5 & J. G. Maessen1

1Department of Cardiothoracic Surgery, University Hospital Maastricht, Maastricht, the Netherlands
2Sanguin Blood Bank Limburg, Maastricht, the Netherlands
3Department of Internal Medicine, University Hospital Maastricht, Maastricht, the Netherlands
4Department of Surgery, University Hospital Maastricht, Maastricht, the Netherlands
5Department of Biochemistry, University of Maastricht, Maastricht, the Netherlands

Background and Objectives In this study we examined whether prestorage leucocyte reduction prevents the accumulation of bioreactive substances in red cell units.

Materials and Methods Measurements were performed in the supernatants of buffy-coat-depleted (standard red cells) and leucocyte-reduced (filtered red cells) red cell units. The effect of storage was evaluated by taking repetitive samples up to 35 days after donation. We determined the concentrations of polymorphonuclear neutrophil (PMN)-derived bactericidal permeability increasing protein (BPI), defensins and annexin A5. In addition, leucocyte counts (using nageotte chamber) were performed on days 0 and 35.

Results During storage, the concentrations of BPI, defensins and annexin A5 in standard red cells gradually increased. However, in the filtered red cells BPI and defensins were found in only a few samples, whereas the annexin A5 concentration in these units did not change during storage. Haemolysis data in both types of red cell components were similar at all time-points, except prestorage. Significant correlations were found between the release of BPI, defensins and annexin A5 into red cell units and the loss of leucocytes during storage.

Conclusions PMNs lose their membrane integrity during cold storage and release their contents into red cell components. Prestorage leucocyte reduction of red cell components prevents the accumulation of BPI, defensins and annexin A5.

Key words: azurophilic granule proteins, leucocyte reduction, red cells.

Introduction

Although the administration of allogeneic blood transfusions is common in cardiac surgery, along with its beneficial effects, allogeneic blood transfusion has been reported to suppress the immune system, thereby contributing to patient morbidity during recovery [1–5]. Known blood transfusion-related complications include human leucocyte antigen (HLA) alloimmunization, non-haemolytic febrile transfusion reactions (FNHTR), platelet refractoriness, transfusion-associated graft-versus-host disease and transmission of infectious agents such as cytomegalovirus, which reside in leucocytes. Because leucocytes have been implicated in the aetiology of these complications, prestorage removal of leucocytes has been advocated to prevent or minimize the incidence of such complications [6]. Although leucocyte filtration of blood transfusion products seems to reduce infectious complications,
certain adverse reactions still occur. Therefore, factors other than intact leucocytes must also play a role in the aetiology of blood transfusion complications.

Previously we and others showed that the plasma supernatant of blood transfusion products contains bioreactive substances that cause febrile reactions [4,7,8]. We showed that bactericidal permeability increasing protein (BPI), originating from the azurophilic granules of leucocytes, is present in red cell units probably as a result of disintegration of polymorphonuclear neutrophils (PMN) during cold storage (4 °C) [9]. In the present study we examined whether prestorage leucocyte reduction prevents the accumulation of those bioreactive substances in red cell units. For this, we measured the levels of BPI, defensins and annexin A5 in 15 buffy-coat-depleted (standard) red cell units and 15 leucocyte-reduced (filtered) red cell units. BPI and defensins originate from the azurophilic granules of leucocytes and are known to be highly toxic [10]. Annexin A5 belongs to a superfamly of phospholipid-binding proteins, the biological functions of which relate to membrane-associated processes [11]. In this study we measured annexin A5 to analyse processes of cell death. Samples were collected prestorage, and 2, 4, 10, 14, 24 and 35 days thereafter, during which period the red cell units were routinely stored at 4 °C. In addition, leucocyte counts were performed on days 0 and 35.

Materials and methods

Preparation of blood components

Whole blood (500 ml) was collected from volunteer donors in primary containers of a ‘top-and-bottom’ triple blood bag system (T2752e; Fresenius Hemocare, Emmer-Compascuum, the Netherlands). Blood collection lasted for maximum time-period of 12 min. The collected blood was placed under cooling plates for at least 4 h, during which period the blood was cooled towards room temperature for further preparations. To prepare standard red cells, whole blood was centrifuged (3704 g for 13 min at 20 °C) (Hereaus Cryofuge 6000 I GMP; Kendro Laboratory Products GmbH, Hanau, Germany). The supernatant (plasma) and the infranatant (red cells) were transferred to integrally attached, secondary satellite containers by using an automatic blood separation device (Compomat G3; Fresenius Hemocare). A total of 30 standard red cell units were suspended in 100 ml of SAG-Mannitol solution and stored for 35 days. By filtration of 15 these standard red cell units with a polyester filter (Bio R 01 BBS max; Fresenius Hemocare), residual leucocytes were removed and filtered red cell units with additive solution were obtained. Filtration was performed within 36 h of donation. Leucocyte counts after preparation and after 35 days of storage were performed using either a cell counter (Onyx; Coulter, Krefeld, Germany), for standard red cell units, or microscopy and a Nageotte chamber, for filtered red cell units [12]. The preparation of red cell units was performed at a controlled room temperature of 20 °C, after which all red cell units were routinely stored in a temperature-controlled refrigerator at 2–6 °C. The red cell units tested in this study were representative of routine preparations. On the final sampling day, all red cell units were checked for bacterial contamination. All were sterile after a culture period of at least 15 days.

Sampling procedure

Samples were taken from all red cell units after the preparation procedure (prestorage), and after 2, 4, 10, 14, 24 and 35 days of storage. Before sampling, the units of red cells were gently agitated for 5 min. By sterile puncture with a long needle through a latex adapter attached to each blood bag, 4-ml samples of were removed at each time-point selected. Samples were collected in evacuated blood collection tubes (4 ml, Monject; Sherwood Medical, Ballymoney, Northern Ireland) containing EDTA. The samples were immediately centrifuged and the supernatant was stored at −80 °C until measurements were performed.

Measurement of BPI

Concentrations of BPI were measured using sandwich enzyme-linked immunosorbent assays (ELISA), as described previously [13]. In brief, 96-well plates (Immuno-Maxisorp; Nunc, Roskilde, Denmark) were coated with human BPI-specific monoclonal antibody 4E3. Free sites were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). Samples and standard dilution series were added for 2 h. Human recombinant BPI (kindly provided by M. Marra; Incyte, Palo Alto, CA) was used for standard titration curves. Washing and dilution buffers contained 80 mm or 40 mm magnesium chloride, respectively, to prevent disturbance by lipopolysaccharide. Biotinylated polyclonal rabbit anti-human BPI immunoglobulin G (IgG) was used as detection antibody. The detection limit for the ELISA was 0.2 ng/ml. Biotinylated antibodies were detected with peroxidase-conjugated streptavidin (Zymed, San Francisco, CA). Finally, 3,3′,5′,5′-tetramethylbenzidine (TMB; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as a substrate. Photometry (450 nm) was performed using a micro-ELISA autoreader. All plasma samples were analysed in the same run.

Measurement of defensins

For measurement of defensins, a commercially available ELISA obtained from HyCult Biotechnology (HBT; B.V., Uden, the Netherlands) was used. The detection limit for the ELISA was 100 pg/ml.

Measurement of annexin A5
Concentrations of annexin A5 were measured by ELISA using a commercially available ELISA kit specific for annexin A5 (Zymutest Annexin A5; Hyphen Biomed, Andrésy, France). Assays were carried out according to the manufacturer's recommendations. The detection limit for the ELISA was 0.5 ng/ml.

Haemolysis measurements
Because annexin A5 is present in low concentrations in erythrocytes [14,15], and thus theoretically could also be released from these cells in the red cell units, we measured the optical density (OD) at all time-points in all units. Haemolysis was monitored by absorption measurements at 405 nm, and is expressed as percentage of total haemoglobin.

Data analysis
Statistical analysis was performed using software (SPSS for Windows, release 9.0; SPSS, Chicago, IL). A repeated-measures analysis of variance was used to compare changes in time between both groups of red cell units. The Mann-Whitney U-test was used to identify significant differences between experimental groups at a single, predetermined time-point. A Wilcoxon matched-pairs signed-ranks test was used for comparisons of values from one variable between two time-points. The correlation between variables was tested by Spearman's rank. Differences were considered significant at P-values of < 0.05. All data are presented as mean ± standard error of the mean (SEM).

Results

Red cell units
The characteristics of the red cell units used are shown in Table 1.

BPI concentration
The BPI concentration in standard red cell units showed a progressive increase during storage (Table 2). The prestorage concentration was 1.8 ± 0.5 ng/ml, and during the storage period BPI concentrations increased to 265 ± 79 ng/ml on day 35, which represented a 150-fold increase. In contrast, BPI was found in only four out of the total of 105 samples of the filtered red cell units. BPI concentrations in these units were 0.1 ng/ml.

Defensins concentration
The defensins concentration in standard red cell units also showed a progressive increase during storage (Table 2). The prestorage defensins concentration was 0.3 ± 0.1 µg/ml, and during the storage period the defensins concentration increased to 8.5 ± 1.6 µg/ml on day 35, which represented a 30-fold increase. In filtered red cell units defensins were found in only eight out of the total of 105 samples, concentrations ranging from 0.10 to 0.21 µg/ml.

Annexin A5 concentrations
Similarly to BPI and defensins, the annexin A5 concentration in standard red cell units also increased progressively during storage (Table 2). The prestorage annexin A5 concentration was 2.3 ± 0.2 ng/ml, and during the storage period increased to 124 ± 22 ng/ml at day 35, which represented a 50-fold increase. In filtered red cells, annexin A5 was detected in low concentrations in all units. Annexin A5 concentrations were 2.3 ± 0.1 ng/ml on day 0 and 3.1 ± 0.3 ng/ml on day 35, and no changes were seen during storage.

Haemolysis
The prestorage OD in standard red cell units was 0.12 ± 0.01, increasing to 0.86 ± 0.07 by day 35 (Table 2). In the filtered red cell units the prestorage OD was 0.14 ± 0.01 and showed an increase to 0.65 ± 0.05 by day 35. The prestorage OD was significantly higher in filtered red cell units than in standard red cell units.

Leucocyte counts
Leucocytes were found in all standard red cell units on day 0 (1.6 ± 0.3 × 10^6/l), and the number of leucocytes had decreased in all of these units by day 35 (to 0.4 ± 0.05 × 10^6/l). Thus, 75% of the original leucocytes were lost during storage. In filtered red cell units leucocyte counts were < 10^6/l in all samples at both time-points, which is more
Correlations between leucocytes and bioreactive substances

The reduction in leucocyte number between days 0 and 35 significantly correlated with the increased concentrations of BPI, defensins and annexin A5 on day 35 (Fig. 1), correlation coefficients being 0.91, 0.95 and 0.86, respectively (P < 0.001 in all cases). Prestorage leucocyte counts in standard red cell units significantly correlated with the day-35 concentration of BPI, defensins and annexin A5, being 0.90, 0.93 and 0.81, respectively (P < 0.001). Furthermore, BPI and defensins concentrations significantly correlated from day 4 (P < 0.001). BPI and annexin A5 concentrations significantly correlated at all time-points (P < 0.01), except for day 0. To exclude erythrocytes as the origin of annexin A5, the OD was measured in all samples. The OD values were similar at all time-points, except for the prestorage sample. In addition, annexin A5 concentrations did not correlate with the OD data at any time-point.

Discussion

This study provides evidence that the accumulation of bioreactive substances in routinely used red cell units can be prevented by prestorage leucocyte reduction. We measured the concentrations of BPI, defensins and annexin A5 in 15 standard red cell units and 15 filtered red cell units. BPI is a human neutrophil granule protein that has been shown to bind to lipopolysaccharide, and exerts bactericidal effects on Gram-negative bacteria and neutralizes the activities of lipopolysaccharide [16]. In red cell units, large amounts of BPI may be released from the azurophilic granules because of PMN activation by the plastic infusion bag or PMN disintegration as a result of cold storage (4 °C) [9]. Defensins
also originate from azurophilic granules of leucocytes and are known to be highly toxic. Annexin A5 was measured to analyse processes of cell death.

Previously we showed that the perioperative increase in BPI plasma levels found in all patients undergoing cardiac surgery was significantly enhanced in patients who intraoperatively received standard red cell units [4]. In the same study we showed that the increase of BPI concentrations in red cell units was related to storage time. In addition, we showed that patients intraoperatively receiving standard red cell units had an enhanced postoperative inflammatory response. These and other [7,12] data suggest that products released from degenerated donor neutrophils in standard red cell units are involved in febrile reactions. Therefore, in the present study we focused on BPI and defensins, both of which originate from PMNs. The low BPI concentrations on day 0 indicate that the blood preparation process activated leucocytes only to a limited extent. The BPI concentrations in standard red cell units progressively increased with storage time (Table 2), which is in accordance with previous observations [4,18]. Willy et al. observed a progressive increase in human neutrophil elastase (HNE) concentrations with storage time in buffy-coat-reduced red cell units [18]. As both BPI and HNE are stored in the azurophilic granule of PMN, and their release is under similar regulatory control [9], the results of Willy et al. [18] are supported by our data. Our results may contribute to the understanding of why age-related factors (possibly the increasing concentrations of bioreactive substances) play a role in red cell transfusion reactions [19].

As for the other markers measured, annexin A5 concentrations increased with storage time in the standard red cell units, but not in the filtered red cell units, suggesting that leucocytes are responsible for the observed increase in annexin A5 concentrations. The type of leucocyte causing this increase remains to be identified. In addition, future studies should reveal the location of annexin A5 in the cell. The clinical implications of defensins and annexin A5 in red cell units also remain to be determined.

Previously, leucocytes present in allogeneic cellular blood components were associated with adverse effects in the recipient [2,3]. Anti-leucocyte alloantibodies in the recipient, and leucocytes in the blood transfusion product, have been considered responsible for most of the FNITRs [20]. In many patients, however, no leucocyte antibodies can be found [21,22] and yet FNITRs occur. Therefore, it is possible that some of the FNITRs occurring after transfusion of standard red cell units may be induced by other substances in these units, e.g. bioreactive substances produced and/or released during storage. As Willy and colleagues [18] have already suggested, elimination of leucocyte products, rather than reduction of viable leucocytes, may contribute to the beneficial effects of leucocyte reduction. Our present data show that prestorage filtering of red cell units not only effectively removed leucocytes but, perhaps even more importantly, was also highly effective at preventing the accumulation of leucocyte-release products during storage. Whether, e.g. bedside filtering [23] of red cell units immediately preceding transfusion, but after cold storage, provides the elimination of leucocyte-release products, which is as effective as prestorage filtering, remains to be elucidated in future studies [24]. The latter may be particularly important for unfiltered red cell units with longer storage times. However, to our present knowledge, prestorage leucocyte reduction of standard red cell units is by far the best way of preventing exposure of recipients to both leucocytes and their release products.

BPI, defensins and annexin A5 were selected as parameters representing a potentially large number of bioreactive substances that can be released from the granules of PMN. Previous work has shown that prestorage leucocyte reduction in red cell units reduces the cytokine interleukin (IL)-1, II-6, II-8 and tumour necrosis factor-α (TNF-α) content during storage [25]. These proinflammatory cytokines [except II-8] and TNF-α have pyrogenic activity and can mediate inflammatory reactions [8,26]. In the present study, leucocyte reduction proved to be effective for preventing accumulation of BPI, defensins and annexin A5 in blood products, as was also shown to be the case for HNE [18]. However, others have shown that prestorage leucocyte-depleted blood still contains non-nucleated leucocyte fragments bearing CD45, CD19 and HLA-DR antigens [27]. However, these researchers used different filters and their preparation of blood products might have been different from the blood-handling procedures described in the present study. Nevertheless, although the concentration of leucocyte fragments was greatly reduced, as opposed to non-leucodepleted blood, these data indicate that prestorage leucocyte reduction may not totally eliminate the risk of transfusion-related complications. Besides prestorage leucocyte reduction, detection and removal of leucocyte residuals appears to be another important step towards safer blood transfusions. In addition, although the substances found in the present study might not have much clinical relevance in low concentrations, the ‘cocktail’ of all markers found (and taking into consideration the bioreactive substances reported to be found in red cell units by others) might be sufficient to trigger all types of adverse reactions, synergistically or additively [28]. It is important to emphasize that the red cell units used in the present study had not been treated any differently from the ones prepared for use in daily clinical practice. This indicates that the products identified in these units are transfused to patients on a daily basis. Hence, future research is an absolute necessity to further improve blood transfusion products.

In conclusion, the preparation process of whole blood towards red cell units does not significantly activate PMNs to release bioreactive substances. Leucocytes lose their
membrane integrity during cold storage and release their contents. Prestorage leucocyte reduction of red cell units effectively prevents the accumulation of these substances.

References


