Cryopreservation of red blood cells: Effect of freezing on red cell quality and residual lymphocyte immunogenicity

A Farrugia, N Shea, S Knowles, R Holdsworth, H Piuronowski, D Portbury, A Romeo

Abstract

Aims—To investigate treatment with glycerol/washing as a potential substitute for freeze-thawing in the production of leucocyte depleted red cell concentrates for patients with a history of non-haemolytic reactions following transfusion.

Methods—The standard procedure of treatment with glycerol –80°C freezing/thawing/washing was compared with a similar procedure in which freezing was omitted. The quality of the resulting red cell products was assessed in relation to: (1) standard red cell biochemical parameters; (2) leucocyte and lymphocyte subset composition using flow cytometry with fluorescent labelled monoclonal antibodies; and (3) immunogenicity of the residual lymphocytes in mixed lymphocyte culture.

Results—Compared with red cells subjected to the standard freeze-thaw technique, red cells undergoing the non-freezing procedure and suspended in additive solutions had significantly better biochemical preservation after 21 days of storage (p < 0·001). Both procedures removed an average 98% of the initial leucocytes at the expense of 18–20% of the red cells. The non-freezing procedure resulted in higher residual concentrations of HLA class II bearing lymphocytes (p < 0·01), but not higher numbers of dendritic cells. Both procedures were equally effective in annulling the residual lymphocytes' ability to act as stimulator cells in one-way mixed lymphocyte culture.

Conclusions—The non-freezing procedure produces a superior product for the provision of red cells to patients with granulocyte antibodies. These products may also offer a lower risk of HLA allo-immunisation to previously unexposed patients.

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Largely superseded by filtration, leucocyte depletion by freeze-thawing has been regarded as the benchmark technique in the provision of red cell concentrates for patients alloimmunised to leucocyte antibodies. The freeze-thaw procedure probably damages the leucocytes by exposing them to the cryoprotectant glycerol, in addition to the damage and loss incurred from the freeze-washing process. This is supported by the finding in this laboratory of 7% of chronically transfused patients experiencing non-haemolytic febrile transfusion reactions (NHFTR) to all but frozen-thawed red cells, despite lower leucocyte burdens being achieved through filtration. The procedure also damages the red cells, however, and an enhancement of the normal storage lesion of red cells at 4°C is observed. Furthermore, –80°C is expensive to maintain.

Following previous reports describing avoidance of NHFTR through the use of glycerol treated and washed red cells, we studied the characteristics of residual red and white cells following glycerol treatment and washing and compared them with units subjected to the standard glycerol treatment/freeze-thawing method.

Methods

Blood units used in this study were 5 days old and were processed to red cell concentrates with leucocyte contents averaging 1650 × 10⁶ a unit. This is in the expected range for red cell concentrates. The somewhat low initial leucocyte content is possibly due to the drop in leucocyte count observed in blood units during storage.

Plasma depleted red cell concentrates were treated with 6·2M glycerol (Glycerolyte 57, Baxter-Fenwal, Deerfield, Illinois, USA) and frozen in a –80°C cabinet freezer (Heterorig CL410, Copenhagen, Denmark), as described by Valeri. After frozen storage (one week for the units described in this study) the units were thawed at 42°C, diluted with 12% NaCl, and then washed with 2 litres of 0·9% NaCl on a Haemonetics 15 cell washer (Haemonetics, Braintree, Massachusetts). The recovered red cell units were centrifuged and the residual saline was removed to give a haematocrit of about 80%. For the purpose of studying red cell storage at 4°C, the cells were then divided into equal volumes and 50 ml of the red cell additive ADSOL2 or 50 ml of the additive AAS³ was added to the cells. Units were then stored at 4°C and sampled at the intervals shown in the results section.

The glycerol treated/washed red cells were prepared in the same way as frozen-thawed red cells, except that after glycerol treatment, the cells were equilibrated for 15 minutes at 22°C. They were then diluted with hypertonic saline and washed as described above.
The final products were also studied as suspensions in the additives described above. Pall RC50 filters (Pall Biomedical, Portsmouth, England) were used to filter red cell units using sterile connection procedures, as described previously.11 Red cell adenosine 5'-triphosphate, extracellular haemoglobin, and extracellular potassium were measured as described before.12 Total red cell counts were performed on a Sysmex E-2500 electronic counter (TOA, Kobe, Japan).

Residual leucocytes were counted flow cytometrically using a method based on that of Dzik et al.13 Following permeabilisation of the leucocytes with Triton × 100, nuclei were treated with RNAase and propidium iodide and then analysed on a FACScan flow cytometer (Beckton-Dickinson, Mountain View, California, USA). Data acquisition was based on dot plots for fluorescence intensity on the propidium iodide channel and intensity of forward scatter. Fluorescence was gated and counted for 10 minutes. A detailed description of the method and its validation will be reported separately.

Phenotyping of the residual leucocytes was also performed on the flow cytometer, using labelled monoclonal antibodies to leucocyte antigens. Reagents were obtained from Becton-Dickinson, and the manufacturer's instructions were followed for sample preparation and analysis. Leucogate is a mixture of fluorescein isothiocyanate (FITC)-labelled anti-CD45, which recognises all leucocytes, and phycoerythrin (PE)-labelled anti-CD14, which recognises monocytes. Analysis on the FACScan required a software package (Simulset) to generate three-part differentials of the residual leucocyte population. The proportion of residual lymphocytes which were HLA class II bearing cells was performed similarly using a mixture of FITC-labelled anti-CD3 (recognising T cells) and PE-labelled anti-HLA-DR (recognising all HLA class II cells). An estimation of the proportion of dendritic cells was performed by similar analysis using PE-labelled anti-CD19 (recognising B cells and dendritic cells) and FITC-labelled anti-CD20 (recognising B cells).

The mixed lymphocyte reaction, using lymphocytes from processed red cell units as stimulator cells, was performed in 96-well microtitre trays: 5 × 10^6 cells per well were inactivated by exposure to 3000 Gray of irradiation from a ^137^Cs source. The cells were incubated for five days with 5 × 10^4 cells from normal donors as responding cells, labelled with ^1^H-thymidine, and further incubated at 37°C before harvesting and counting on a liquid scintillation spectrometer (Packard Model 2450).

Statistical analysis was performed using a computer package (Statgraphics, Statistical Graphics Corporation, Maryland, USA) to analyse data. P values and confidence intervals for differences were assessed with the unpaired t test, and were considered significant at p < 0.05.

### Results

Table 1 summarises data for extracellular haemoglobin and potassium in red cell suspensions at day 21. In non-frozen units, both variables were significantly lower, indicating that this process was less damaging to the red cells. Both procedures resulted in a red cell depletion of 5–25% while removing about 98% of the leucocytes. There was no significant difference between the two groups for these two measurements.

The leucocyte and lymphocyte subset distribution for the two processes is shown in table 2. Both methods resulted in a preferential removal of granulocytes. Phenotyping the residual lymphocytes showed that the freezing procedure resulted in significantly lower concentrations of HLA class II bearing cells (difference between means −7.25; 95% confidence interval −1.86 to 12.64). Dendritic cells were depleted to similar levels by both methods. With filtration, residual leucocyte numbers were significantly lower (p < 0.01) than for both the other leucocyte depletion procedures. A higher proportion of granulocytes passed through the filters, however, resulting in residual granulocyte numbers of about 4.4 × 10^6/unit compared with approximately 0.54–0.74 × 10^6/unit for the other procedures.

The figure shows the effect of the two methods on the ability of lymphocytes in the red cell concentrates to act as stimulator cells in the mixed lymphocyte reaction. Both procedures severely and equally destroyed lymphocyte stimulation of two different responding cells.

### Table 1 Extracellular haemoglobin and potassium concentrations in red cell suspensions 21 days after processing

<table>
<thead>
<tr>
<th>Method</th>
<th>Free Hb mg/l</th>
<th>Free K+ mEq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol treatment/washing (n = 4)</td>
<td>730 (31)</td>
<td>7.6 (11)</td>
</tr>
<tr>
<td>Glycerol treatment/freezing/washing (n = 5)</td>
<td>3730 (950)</td>
<td>30.3 (23)</td>
</tr>
<tr>
<td>Difference between means</td>
<td>−3000</td>
<td>−22.7</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>−4160–1850</td>
<td>25.6–20</td>
</tr>
</tbody>
</table>

### Table 2 Types of leucocytes in red cell concentrates

<table>
<thead>
<tr>
<th>Product</th>
<th>Total leucocytes × 10^6/unit</th>
<th>Lymphocytes as % total leucocytes*</th>
<th>Granulocytes</th>
<th>Monoocytes</th>
<th>HLA class II cells</th>
<th>Dendritic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>1650 (388)</td>
<td>32 (10)</td>
<td>64 (8)</td>
<td>4 (3)</td>
<td>17 (7)</td>
<td>ND</td>
</tr>
<tr>
<td>Glycerol/ washed</td>
<td>268 (9–2)</td>
<td>95 (2)</td>
<td>2 (1)</td>
<td>2 (2)</td>
<td>11 (4)</td>
<td>0.13 (1.1)</td>
</tr>
<tr>
<td>Glycero/ frozen/washed</td>
<td>37.8 (316)</td>
<td>98 (2)</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>3.8 (1)</td>
<td>1.83 (2.9)</td>
</tr>
<tr>
<td>Filtered</td>
<td>110 (9–6)</td>
<td>59 (11)</td>
<td>40 (9)</td>
<td>0</td>
<td>27 (7)</td>
<td>0</td>
</tr>
</tbody>
</table>

*n = 7, fn = 4; all results show mean (SD).
Discussion

The indications for frozen-thawed red cells have been comprehensively reviewed. The maintenance of a frozen rare red cell bank is important, but leucocyte depletion to avoid NHFTR and to prevent alloimmunisation to HLA antigens has always been our organisation’s main reason for providing these products. Over 90% of our frozen cell issue is for chronically transfused red cell recipients. These patients exhibit all the manifestations of NHFTR following transfusion of other red cell products, including products leucocyte depleted by other means. The present investigation was initiated when the increased sensitivity of the flow cytometric counting method showed that red cells rendered leucocyte poor by currently available polyester filters resulted in lower residual leucocyte numbers than frozen-thawed red cells, despite the latter being better tolerated by the patients described above. The freezing procedure seemed to be reducing the residual leucocytes’ ability to cause a reaction. The demonstration of a selective removal of granulocytes shown by previous work and by the present study (table 2) when red cell concentrates are frozen-thawed may explain this, as the highest incidence of NHFTR has been described in patients with antibodies to granulocytes. Because it has been shown that polyester filtration permits relatively more granulocytes in the product, this may result in reactions in these patients which are avoided when transfusing the granulocyte-poor frozen cells.

This contention is supported by the data for filtered cells shown in table 2 which confirm a higher absolute granulocyte content in filtered cells compared with frozen or glycerol treated cells, despite a lower total leucocyte content. The filters we used at the time of this study included the ones described in table 2; we have subsequently studied filters with higher leucocyte and granulocyte-depletion efficiency (Farrugia et al; unpublished observations).

Similar levels of leucocyte removal as well as a similar leucocyte subset composition were attained when the red cell concentrates were subjected to an identical procedure without freezing. This confirms previous findings that this procedure is equally effective in removing leucocytes and preventing NHFTR. The exposure of leucocytes to glycerol results in extensive damage of the cell membrane, nuclear changes, and leakage of nuclear material from the cell, causing leucocyte clumping and removal of the resulting aggregates during subsequent cell washing. The freeze-thawing procedure also substantially damages the red cells. Our study shows that red cell damage, as assessed by haemolysis and potassium leakage is significantly decreased when red cells are treated with glycerol but not frozen. This suggests that with the use of special washing harnesses to enable all procedures to be performed in a closed system, this method will allow better quality red cells to be delivered and with fewer logistical difficulties than frozen-thawed red cells.

Analysis of the subset composition of the residual lymphocytes in the processed red cells showed that the freezing procedure resulted in significantly lower numbers of HLA class II bearing cells. Class II positive antigen presenting cells are responsible for initiating the immune response which results in HLA antibody formation. It might therefore be expected that glycerol treated washed cells would pose a higher risk of inducing HLA alloimmunisation than frozen-thawed cells. The finding that the two procedures produced equivalent numbers of dendritic cells, considered to be the most important in HLA alloimmunisation, suggests, however, that their immunogenic potential should be similar. Further evidence of the non-immunogenicity of the residual lymphocytes in both procedures was supplied by the reduction in their ability to stimulate responder cells in the one-way mixed lymphocyte reaction (figure). This test has already been used as an in vitro indicator of the ability of passenger lymphocytes in blood products to cause an immune response and HLA antibody formation when transfused. The similarly reduced level of incapacitation in this test suggests that the residual lymphocytes from both will be incapable of causing HLA alloimmunisation. Patients not previously immunised may therefore be kept in this state when transfused with these products, thereby.
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avoiding subsequent complications such as unresponsiveness to platelet transfusions, should these be required.

In summary, omitting the freezing step in the production of leucocyte depleted red cells by freeze-thawing results in a product with improved red cell quality. The analysis of the leucocyte and lymphocyte subsets suggests that this product should also avoid NHFTR and HLA alloimmunisation. If −80°C freezing could be avoided this improved product could also be delivered at lower cost.

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2 Crowley JP, Valeri CR. The purification of red cells for transfusion by freeze preservation and washing I. The mechanism of leucocyte removal from washed, freeze-preserved red cells. Transfusion 1974;14:188–95.
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