**CONCISE REPORT**

**The Effect of the Plasticizer Di(2-ethylhexyl)phthalate on Red Cell Deformability**

By R.S. Labow, R.T. Card, and G. Rock

Red cell concentrates (RCC) are stored for 35 to 42 days in plastic containers manufactured with the liquid plasticizer di(2-ethylhexyl)phthalate (DEHP). DEHP leaches from the polyvinylchloride (PVC) plastic bag, then binds to and stabilizes the RC membrane. This study was undertaken to determine the deformability of the RC membrane using an osmotic gradient ejectactometry and to relate these measurements to the concentration of DEHP in the stored RCC. Pooled RCC was aliquoted into PL146 (PVC), PL732 (polyolefin), and PL732 (with added DEHP) bags with samples removed weekly for analysis of osmotic fragility, deformability, and DEHP concentration. The adenosine triphosphate (ATP) content was also measured. The increase in osmotic fragility during storage was greater when RCC was stored without DEHP. In addition, there was a decrease in the maximum elongation index (El max) when there was decreased DEHP in the storage bag. The osmolarity (Omax) at which El max occurred, as well as the Omin, the osmolarity at which minimum elongation (El min) occurred was higher in the PL732 container than in the PL146 or in the PL732 to which DEHP had been added. These changes could be reversed by addition of DEHP at the beginning of the storage period, showing a direct correlation between DEHP concentration during storage and RC membrane flexibility. By a better understanding of the mechanism of DEHP protection, it might be possible to substitute a less toxic stabilizing compound.

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directly with DEHP concentration during storage.\textsuperscript{15,16} In order to try to elucidate the mechanism of the DEHP protection, we measured the RC deformability using the osmotic gradient ektacytometer and correlated this to the DEHP concentration during storage and have related the characteristic measurements (Omin, Ei max, Omax) to the osmotic fragility and adenosine triphosphate (ATP) content measured on the same samples.

MATERIALS AND METHODS

Preparation of RC concentrate. Whole blood was collected from O-positive donors who met all the criteria of the Canadian Red Cross Blood Transfusion Service. RC concentrate (RCC) was prepared using standard blood bank procedures. RCC (from two donors) was pooled and aliquoted by weight into three containers: the PL146 bag (PVC standard RCC storage container that is routinely used), the PL732 (polyolefin container normally used for platelet concentrate storage) to which dimethylsulfoxide (DMSO) was added, and the PL732 container with added DEHP dissolved in the same amount of DMSO. There was also a PL732 container used with no additions, and a PL146 container with DMSO added as additional controls. The DEHP was dissolved in DMSO to give a final concentration of 150 \( \mu \text{g/mL} \) RCC. A stock solution of DEHP in DMSO (100 mg/mL) was prepared. DEHP 30 to 40 mg was added to give a final concentration in each unit of RCC of 150 \( \mu \text{g/mL} \). The amount added was adjusted to the exact volume of RCC in the container. These three containers were stored under standard conditions. On days 0, 7, 14, 21, 28, and 35 a sample was removed from each container using sterile technique and analyzed for osmotic fragility using the method of Roy and Noteboom.\textsuperscript{17} The DEHP concentration was measured by high performance liquid chromatography (HPLC) using a modification of a previously published method.\textsuperscript{18,19} This study was performed in triplicate, ie, three pools of two donors for each pool were aliquoted into nine containers using the three conditions described. Since the determination for each data point was done in duplicate, the number of observations was six. This includes the ektacytometry measurements as well. All plastic containers were obtained from Fenwal Corporation, Deerfield, IL. The ATP concentrations were measured on each sampling day, according to the procedure of Beutler.\textsuperscript{20}

Measurement of RC deformability. Part of the sample removed on days 0, 7, 14, 21, 28, and 35 (1.0 mL) was placed in a small plastic tube, packed in ice, and shipped by air at 4\( ^\circ \text{C} \) to Saskatoon, where the deformability measurements were performed the same day using a Technicon ektacytometer (Technicon Instruments Corporation, Tarrytown, NY) in the osmocan mode. In addition, RCC was prepared and stored under standard conditions at the Saskatoon Center and the results of RC deformability compared with the RC samples that were shipped by air from Ottawa. The method consists of subjecting RC in 3.1% polyvinylpyrrolidone (PVP) to a constant fluid shear stress over an osmolality gradient from 60 to 600 mosm/kg prepared using phosphate-buffered saline. The resulting deformation was then measured and recorded continuously. The EI is a measure of RC deformability and varies with the osmolality of the suspending medium. The characteristic osmotic profile is defined by a number of indices: Ei max is the maximum elongation that occurs in iso-osmolar medium (Omax); Ei min is the lowest deformability that occurs in hypotonic medium (Omin).

RESULTS

The DEHP concentrations in each of the storage containers changed as was expected. The variation in the DEHP concentration (Table 1) is due to variation in the amount by

<table>
<thead>
<tr>
<th>Day</th>
<th>PVC</th>
<th>PO Plus DMSO</th>
<th>PO Plus DEHP in DMSO</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>22 ± 1.1</td>
<td>2.7 ± 1.4</td>
<td>155 ± 57</td>
</tr>
<tr>
<td>7</td>
<td>54 ± 19</td>
<td>4.2 ± 1.4</td>
<td>95 ± 10</td>
</tr>
<tr>
<td>14</td>
<td>89 ± 34</td>
<td>6.0 ± 2.1</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>21</td>
<td>116 ± 54</td>
<td>7.4 ± 3.2</td>
<td>85 ± 11</td>
</tr>
<tr>
<td>28</td>
<td>141 ± 77</td>
<td>8.4 ± 4.0</td>
<td>78 ± 17</td>
</tr>
<tr>
<td>35</td>
<td>174 ± 88</td>
<td>9.2 ± 4.5</td>
<td>66 ± 12</td>
</tr>
</tbody>
</table>

Fig 1. RC deformability measured in RCC stored in three types of bags: PL146, PL732 plus DMSO, and PL732 plus DMSO and DEHP. (A) Day 0; (B) day 35.
in a PL732 container to which nothing was added. This was another check that all observed effects were related to DEHP concentration and not to the DMSO, with the only differences observed being related to the DEHP concentration.

Figure 1 shows the RC deformability for RC storage under all three storage conditions. Using the student's $t$ test, there was a statistically significant difference between PL732 plus DMSO alone compared with PL146 or PL732 plus DMSO and DEHP; this can be clearly seen when the El max, Omax, and Omin values are plotted separately (Fig 2) with error bars. This profile was identical for all three storage conditions on day 0 (Fig 1A). The El varied with the osmolarity of the suspending medium and El max occurred in iso-osmolar medium (290 mosm/kg, Omax) as was expected. El min occurred at 140 mosm/kg (Omin). Figure 1B shows the deformability profiles of the same samples on day 35 of storage. The storage container with no DEHP had a greater loss of deformability El max (Fig 2A). Omax values (Fig 2B) were shifted to the right, that is, toward higher osmolality. Addition of DEHP to the non-DEHP bag partially corrected the defect. With DEHP present from the start of the storage period there was a reversal Omin shift to higher osmolality (Fig 2C). The Omin values correlated directly with the 50% hemolysis point on the osmotic fragility curves (Fig 3). The data points for the osmotic fragility curves represent the mean of the values obtained from three pools. The standard deviation was calculated for the concentration of sodium chloride at 50% hemolysis for each pool for each sampling day (Table 2). There was no statistically significant difference between the PVC or the PO container in the 50% hemolysis point up to day 21 of storage. After day 21, it was evident that the increased concentration of DEHP caused a decrease in the osmotic fragility. The shift to higher percent sodium chloride was equal to the Omin shift on the deformability profiles on day 35 of storage.

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**Fig 2.** The variation of El max (A), Omax (B), and Omin (C) with increasing storage time in three types of storage containers: PL146, PL732 plus DMSO, PL732 plus DMSO and DEHP.

**Fig 3.** The variation of osmotic fragility with increasing storage time in three types of containers: PL146, PL732 plus DMSO, and PL732 plus DMSO and DEHP. Day 0, O; day 21, □; and day 35, △.
The ATP concentration in the RCC decreased with increasing storage time to the same extent in all three types of storage conditions (Fig 4). The differences observed were not statistically significant (student’s t test).

**DISCUSSION**

Over the past few years, concern has arisen about the toxic effects of DEHP and its metabolites. We\(^{11}\) and others\(^{16,17}\) have shown that DEHP binds to the RC membrane and lowers its osmotic fragility. When attempts were made to store RCC in non-DEHP containers beyond 21 days, increased osmotic fragility and plasma-free hemoglobin were measured\(^{23}\) as well as decreased in vivo survival times.\(^{14}\) Horowitz et al\(^{10}\) stored RCC in polyethylacrylate bags and showed that RC metabolism was preserved, but the RC membrane had greatly increased fragility. We have studied the changes in RC deformability during storage and have shown that DEHP altered the membrane flexibility. The presence of DEHP enhanced RC deformability and resulted in a more “flexible” cell after prolonged storage. The fact that we observed no difference in ATP levels with different storage conditions (Fig 4) suggests that DEHP exerted its effect directly on membrane flexibility and not on RC function. Decreased deformability is directly related to loss of membrane surface area, which is due to the altered cholesterol to lipid ratio.\(^{6}\) The protection by DEHP may be due to the incorporation of the very hydrophobic DEHP into the area that was vacated by the lipid, alternatively the DEHP may help by bridging the asymmetric bilayers. By a better understanding of the mechanism of this DEHP protection, it might be possible to introduce some other stabilizing lipophilic compound that would not be as toxic as DEHP.

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**REFERENCES**

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