Plasma-Mediated Alterations of Erythrocyte Deformability by Perfluorochemical Blood Substitutes

By Gretchen M. Holloway, E.A. O’Rear, and B.M. Fung

The effect of perfluorochemical blood substitutes (eg, Oxypherol or Fluosol-DA) on red cell deformability was investigated because these emulsions are in direct contact with red cells when they are used as temporary circulatory aids. Erythrocyte deformability was assessed by a constant volumetric flow rate filtration method. The results of in vitro incubation experiments indicate that perfluorotributylamine causes the deformability of human red cells to decrease significantly in the presence of plasma. However, there is no obvious loss in the deformability when washed cells are used. Neither mean cell volume nor white cells appear to be responsible for the observed effects of perfluorotributylamine. Perfluordecalin and perfluorotributylamine, two perfluorochemical compounds that are widely applied clinically, do not induce significant changes in red cell deformability with or without plasma. These results indicate the need for in vitro testing in the development of perfluorochemicals as blood substitutes.

© 1986 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Erythrocytes were obtained from fresh human blood donated by healthy, nonsmoking male volunteers from whom prior informed consent had been obtained. Whole blood was used directly for some incubations, but in each case, red cells were resuspended in Ringer’s/human albumin before the deformability test. Erythrocyte samples were washed twice each with normal saline and then Ringer’s solution. Washing involved centrifugation, removal of supernatant, and resuspension of the cells in order to remove plasma and most of the white blood cells. Finally, the washed cells were resuspended in a solution appropriate for the ensuing procedure.

Erythrocyte deformability was measured using a constant volumetric flow rate filtration technique, in which pressure is used as an indication of the flow resistance through the 3-μm pores of a Nuclepore filter. The pressure v filtration time curve becomes linear with a slight positive slope after a short transient period; an initial pressure drop (P0) is obtained by extrapolation of the linear region back to zero time when pumping by contaminant white cells or thrombi does not occur. Tests in this study were at a volumetric flow rate of 2 mL/min and hematocrit of 3% to 4% in Ringer’s/human albumin. Details of the filtration method have been described previously.18,19 Cell counts for each sample were adjusted with a Boehringer-Mannheim HC-333 cell counter (Houston); the hemato-

From the Departments of Chemical Engineering and Chemistry, University of Oklahoma, Norman.

Supported in part by the American Heart Association and its Oklahoma Affiliate and by Public Health Service grant No. HL32640. G.M.H. gratefully acknowledges receipt of a Graduate Professional Opportunities Program Fellowship.

Submitted March 8, 1985; accepted July 18, 1985.

Address reprint requests to Prof E.A. O’Rear, 202 W Boyd, Room 23, Norman, OK 73019.

© 1986 by Grune & Stratton, Inc.

0006-4971/86/6701-0026$03.00/0

crit was determined in duplicate by the microhematocrit method. Cell morphology was checked by light microscopy of wet mounts.

In order to investigate the mode by which the PFCs may affect the deformability of erythrocytes, the blood samples were treated in several ways:

1. The erythrocytes were harvested immediately after the blood was drawn, resuspended in a 0.05 g/L Ringer’s albumin (human) solution, and then incubated with Oxypherol.
2. Whole blood was incubated with Oxypherol, or another PFC emulsion to be tested, before the erythrocytes were separated.
3. White cells, for the most part, were removed from the whole blood; the recombined plasma and erythrocyte suspension was subsequently incubated with Oxypherol.
4. The plasma was first incubated at 56°C for one hour to denature the proteins; then it was centrifuged to remove the flocculated proteins; finally, the cells were resuspended in the treated plasma and mixed with Oxypherol before incubation.

Samples were washed and the PFCs removed before the filtration tests. Oxypherol and Fluosol-DA emulsions and annex solutions were obtained from Alpha Therapeutics Corporation and prepared according to instructions. Except where noted, incubations were at 37°C for four hours; the total time for workup, incubation, and measurements was less than eight hours in all experiments. PFC emulsions were added to 10 vol%, roughly equivalent to the transfusion of 1 unit. In each case, a control was run with cells prepared in a similar manner but without the PFC emulsion.

RESULTS AND DISCUSSION

When Oxypherol was added to washed erythrocytes, with incubation, it did not affect the deformability of the erythrocytes (Table 1, part A). However, when Oxypherol was first incubated with whole blood (Table 1, part B) or plasma (Table 1, part C), it caused the erythrocytic deformability to decrease significantly (Gosset’s t test). To confirm that the flow properties had been altered, viscosities of red cell suspensions (70% hematocrit) obtained from paired whole blood samples incubated four hours with and without Oxypherol were measured in a capillary tube viscometer (1.15-mm ID). At wall stress \( r_w = 2.99 \text{ dyn/cm}^2 \), suspension viscosity increased after incubation with Oxypherol (controls, \( 13.5 \pm 2.6 \text{ cp (5)} \); Oxypherol, \( 16.2 \pm 4.4 \text{ cp (5)} \); \( P < .15 \)). A high hematocrit was used in these tests because, according to Dintenfass,29 red cell “rigidity” becomes a significant factor for viscosity only at higher cell concentrations. The mean cell volume (MCV) was calculated, and unlike previous results for canine cells, no changes due to experimental procedures were observed (Table 2), although the MCV varied slightly among individuals. Results for MCV compared favorably with reference values for normal cells.31,32 Resuspended in Ringer’s/albumin, red cells for all samples exhibited normal morphology. The deformability tests indicate that one or more of the components in the plasma causes the Oxypherol to interact with the erythrocytes. When the plasma was first incubated at 56°C for one hour (and the denatured proteins were removed before being incubated with the red cells and Oxypherol), the effect of the PFC emulsion on the erythrocyte deformability was reduced (Table 1, part D). However, there was also an apparent increase in the erythrocytic deformability (ie, the pressure drop decreased) of the denatured-plasma control compared with the whole blood control. As a result, a difference between the control and the sample treated with Oxypherol was still observed in this case; but unlike samples incubated with undenatured plasma, this difference was not statistically significant. Therefore, we speculate that the decrease in the red cell deformability may be due to plasma protein-mediated transfer of the PFC component in Oxypherol to the membrane of the erythrocyte. The validity of this hypothesis and the component responsible for the transport of the PFC remains to be tested. Alternatively, the particular mechanisms of reduced deformability might be enhanced platelet activation33 or protein adsorption. The former of these two is particularly suspect, since a recent report indicates that platelet activation can affect red cell deformability.24 Additional studies are planned to investigate these mechanisms. Because results for recombined plasma with Oxypherol were similar to those for whole blood with Oxypherol, it appears that white blood cells are not responsible for the observed changes (Table 1, parts B and C).

### Table 1. Results of Erythrocyte Deformability Measurements

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition</th>
<th>Initial Pressure Drop (mm Hg) ± SD</th>
<th>Statistically Significant Difference (( P &lt; .01 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Prewashed cells</td>
<td>Control</td>
<td>21.2 ± 1.5 (5)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>21.8 ± 2.3 (5)</td>
<td>No</td>
</tr>
<tr>
<td>B. Whole blood</td>
<td>Control</td>
<td>19.2 ± 2.7 (12)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>26.0 ± 3.8 (12)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Oxypherol ± PC</td>
<td>27.0 ± 2.9 (4)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>PFTBA ± PFD</td>
<td>28.4 ± 1.9 (4)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Fluosol-DA</td>
<td>21.9 ± 0.2 (3)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Pluronic F-68</td>
<td>18.5 ± 1.2 (4)</td>
<td>No</td>
</tr>
<tr>
<td>C. Plasma</td>
<td>Control</td>
<td>19.3 ± 3.4 (6)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>24.9 ± 1.3 (6)</td>
<td>Yes</td>
</tr>
<tr>
<td>D. Denatured plasma</td>
<td>Control</td>
<td>14.8 ± 5.1 (5)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>18.6 ± 8.8 (5)</td>
<td>No</td>
</tr>
</tbody>
</table>

A larger pressure drop of a sample compared with the control indicates a loss in deformability. The last column shows whether or not the difference between the sample and the corresponding control is statistically significant within a 99% confidence limit. PC, phosphatidylcholine; PFTBA, perfluorotributylamine; PFD, perfluorodecalin.
Previous workers have related toxicity of some PFC emulsions to particle size. Electron microscopy results show that, when prepared by established methods, the average particle diameter of perfluorotributylamine (PFTBA)-Pluronic F-68 emulsions corresponds to a safe value for infusion.25 Similarly, the mean particle size for perfluorodecalin–perfluorotributylamine (PF–PFTBA) and Pluronics F-68 emulsions is reported to be 0.1 μm.26,27 According to literature supplied by Alpha Therapeutics, Oxypherol and Fluosol-DA emulsions have an average particle size of 0.1 to 0.2 μm. Using a centrifugation method,28 we obtained mean particle sizes of less than 0.12 μm for Fluosol-DA and of 0.25 μm for Oxypherol.

Because we found that the Oxypherol decreases the deformability of erythrocytes, it is important to examine whether or not other PFC blood substitutes have a similar adverse effect. The PFC in Oxypherol is PFTBA (20.0%) and the emulsifier is Pluronic-F68 (2.56%). Because of the long retention time of PFTBA in the liver and spleen, it is generally used for animal tests but not for clinical purposes. The PFC preparation that has been tested extensively in Japan and China and to a lesser degree in the United States is Fluosol-DA, 20%. Its PFC components are 14% PF and 6% PFTPA, while 0.4% yolk phosphatidylcholine (PC) and 0.032% potassium oleate are used as co-emulsifiers in addition to 2.7% Pluronic-F68. The other components (3.0% ethyl starch as an oncotic agent, 0.60% NaCl, 0.034% KCl, 0.020% MgCl₂, 0.08% CaCl₂, and 0.21% NaHCO₃ as osmotic and buffering agents, and 0.18% glucose) are identical in the two types of emulsions. Fluosol-DA also has 0.80% glycerol for viscosity control, but this is not likely to affect the results as discussed below.

When Fluosol-DA was used to replace Oxypherol, it did not show a statistically significant alteration in erythrocytic deformability (Table 1, part B), in light of the findings for Oxypherol, this was an important negative result. This could be due to the presence of PC or a difference in the PFCs. To examine whether or not PC protects erythrocytes against the action of Oxypherol, we added the phospholipid to Oxypherol, sonicated the system to ensure homogeneity, and tested the emulsion in the incubation system. The results were not different from those of Oxypherol alone (Table 1, part B).

Table 2. Mean Cell Volume After Incubation for Four Hours at 37 °C

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition</th>
<th>MCV (μm²) ± SD</th>
<th>Statistically Significant Difference (P &lt; .01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prewashed cells</td>
<td>Control</td>
<td>81.7 ± 11.4 (3)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>88.8 ± 6.2 (3)</td>
<td>No</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Control</td>
<td>88.9 ± 4.8 (11)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>92.2 ± 6.4 (10)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Oxypherol + PC</td>
<td>89.9 ± 3.0 (4)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>PFTBA + PFD</td>
<td>89.4 ± 1.2 (4)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Fluosol-DA</td>
<td>90.1 ± 4.2 (3)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Pluronic F-68</td>
<td>88.4 ± 2.8 (4)</td>
<td>No</td>
</tr>
<tr>
<td>Plasma</td>
<td>Control</td>
<td>84.3 ± 5.4 (6)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>87.1 ± 8.7 (6)</td>
<td>No</td>
</tr>
<tr>
<td>Denatured plasma</td>
<td>Control</td>
<td>85.6 ± 3.8 (5)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxypherol</td>
<td>90.0 ± 3.6 (5)</td>
<td>No</td>
</tr>
</tbody>
</table>

Therefore, the decrease of the erythrocyte deformability must be due to different properties of the different PFCs. To ascertain this point, we made another emulsion that had the same composition as Fluosol-DA, except that PFTPA was replaced by PFTBA. This emulsion decreased the deformability of erythrocytes in the same way as Oxypherol (Table 1, part B). Finally, the emulsifier Pluronic F-68 alone was tested to make sure that it was not the cause of the decrease in erythrocytic deformability. No adverse effect was found (Table 1, part B). This negative result was not a consequence of different batches of Pluronic F-68 because the same material was used in the PFTBA + PFD experiments. Moreover, the mean particle sizes above indicate that Oxypherol has less interfacial area available to the surface agent Pluronic than does Fluosol-DA. If mass transport of Pluronic were important in affecting the erythrocytic deformability, a smaller surface area would have less effect. This is contrary to what was observed.

From the results shown in Table 1 and the above discussion, we conclude that PFTBA causes the deformability of human erythrocytes to decrease considerably in the presence of plasma, whereas PFTPA and PFD have comparatively little effect. Many techniques have been used to assess erythrocyte deformability, although the sensitivity of various testing methods relative to the physiological deformation requirements has not been clearly established. However, at least one clinical trial with Fluosol-DA is consistent with observations of this study.12,13

The results of these experiments have an important implication beyond the loss of patient red cell mass because it may be related to other biological effects of the PFCs, such as their different retention times in the body and different cytotoxicities. It has recently been reported that a 25% emulsion of PFTBA is selectively cytotoxic to macrophages,29 but it is not clear whether PFTPA and PFD have a similar effect. It is of interest to note that the effect of Oxypherol (an emulsion of PFTBA) on neutrophils29 is much larger than that of Fluosol-DA (which contains PFTPA and PFD). Through the use of nuclear magnetic resonance, electron microscopy, and differential scanning calorimetry, we have also found that the physical state of PFTBA in phospholipid bilayers is quite different from those of the
other two PFCs. Fortunately, PFTBA preparations are not used for clinical purposes. We suggest that in vitro tests, such as cytotoxicity, erythrocytic deformability, and blood compatibility, of these and other PFCs continue to be important to their development in clinical applications and should be

REFERENCES

Plasma-mediated alterations of erythrocyte deformability by perfluorochemical blood substitutes

GM Holloway, EA O'Rear and BM Fung