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Assessment of erythrocyte shape by flow cytometry techniques

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Background: Red blood cell (RBC) rheology is altered in different diseases, including acute conditions such as patients in intensive care units (ICU) with sepsis or with an inflammatory reaction due to postoperative states or intracerebral haemorrhage, or chronic conditions such as diabetes mellitus or terminal renal failure. Several techniques are available to assess alterations in RBC rheology, especially deformability, but they are too cumbersome to be used on a large number of cells.

Objective: To develop a new, rapid flow cytometry technique for easy assessment of RBC shape in patients.

Methods: In flow cytometry, healthy human RBC shape shows a bimodal distribution related to the biconcave form. On this histogram, the second Pearson coefficient of dissymmetry (PCD) representing the asymmetry of this histogram and the spherical index (M2:M1) were calculated, both representing the spherical shape. This technique was used in healthy volunteers (n = 17) and in diseases characterised by abnormalities in RBC rheology, including terminal renal failure requiring haemodialysis (n = 28), diabetes mellitus (n = 18), sepsis (n = 19) and acute inflammatory states (postoperative, intracerebral haemorrhage, chronic obstructive pulmonary disease, epilepsy or severe drug intoxication; n = 21). Multivariate analysis was performed to determine the factors influencing RBC shape.

Results: Measurement of RBC shape was highly reproducible. A good correlation was observed between the PCD and the spherical index, except in the critically ill patients without sepsis. RBCs were more spherical in patients with terminal renal failure (PCD -0.56 (0.14), p < 0.05), diabetes mellitus (PCD -0.59 (0.23), p < 0.05), sepsis (PCD -0.58 (0.22), p < 0.05) or an acute inflammatory state (PCD -0.65 (0.29), p < 0.05) than in healthy volunteers (PCD -0.89 (0.12)). The spherical index was also increased in all populations compared with healthy volunteers (terminal renal failure 2.30 (0.20); diabetes mellitus 2.27 (0.38); sepsis 2.28 (0.37); acute inflammatory state 2.35 (0.42) vs healthy volunteers 2.72 (0.47); all p < 0.05). Multivariate analysis demonstrated that the underlying pathology (sepsis, acute inflammatory state, diabetes mellitus, terminal renal failure) was the principal cause of these RBC shape abnormalities.

Conclusion: RBCs are characterised by an increased spherical shape in many disease states. The measure of the second PCD in flow cytometry is a new, easy method to investigate RBC shape in various diseases. This technique could facilitate the investigation of abnormalities of RBC rheology.
Patients and Methods

Patients

Following approval by the André Vésale Hospital Ethics Committee, this study included 17 healthy volunteers (hospital employees of both genders), 18 patients with insulin-dependent diabetes, 28 patients with terminal renal failure requiring iterative haemodialysis (but no diabetes) and 40 critically ill patients with and without sepsis, and demonstrated a good correlation between these two indices.10 To validate this technique, we extended the flow cytometry studies to other diseases known to induce RBC rheology abnormalities including diabetes mellitus11 and terminal renal failure requiring haemodialysis.12

Methods

Study protocol

In all subjects, blood samples were taken once and collected in EDTA tubes (2.5 ml of blood in 0.06 ml EDTA, 0.235 mol/l, Venoject, Terumo, Leuven, Belgium). For patients with terminal renal failure, blood samples were taken before haemodialysis to exclude a rheological effect of this procedure.15 In patients in the ICU, blood samples were drawn during the first 24 h of sepsis (in septic patients) or during the 24 h after ICU admission.

Blood analysis

Blood analysis included RBC count, haemoglobin concentration, mean corpuscular volume, leucocyte count, blood glucose, C reactive protein, sodium and urea concentrations; plasma osmolality was calculated. Blood lactate was measured in patients in the ICU and haemoglobin subtype 1c (HbA1c) in patients with diabetes.

Analysis of RBC shape

Data were collected on a Becton-Dickinson FAC Scan calibur cytfluorimeter (Becton-Dickinson, San Jose, California, USA). RBC samples were analysed within 90 min after blood sampling. The forward light scatter channels (FSCs) were set on lineal gain. Cell size is the principal component of the FSC signal. Both the general principles of the FACScan flow cytometer and the details used herein have been described elsewhere.16 17 For estimation of RBC shape, we expanded the technique described by Rolles-Curl et al10 using the FSC signal in iso-osmolality, applying low shear stresses (12 μl/min RBC flow rate) to allow the RBCs to rotate in the flow18 without deformation due to shear stresses, as described by Cokelet and Goldsmith.19 Moreover, we did not add fluorescently labelled agglutinins that may alter RBC shape.

Whole blood (2 μl) was mixed with isotonic (286 mM Osm) phosphate-buffered saline at 25˚C. The study was limited to 15 000 events and lasted for 15 s. In healthy volunteers, after the position of the obscuration bar was modified, the FACScan clearly viewed the flow of ellipsoid, biconcave RBCs as essentially two populations of cells and the FSC histograms showed a typically bimodal distribution of RBCs (fig 1).16 17 We fixed two gates of interest for these histograms: R1 and R2 (fig 1). The limit between R1 and R2 was the mean value in the valley between the two modes for the volunteer population. Two median values (M1 and M2) were calculated for each predetermined gate of interest (R1 and R2).

The M2:M1 ratio, termed the spherical index, and the second PCD20 (asymmetry of the global histogram = s(mean−median)/s) express the sphericity of the RBC. A negative PCD (which indicates a left asymmetry) and a decrease in M2:M1 correlate with the sphericity of the RBC. The cytometer settings were kept constant and the stability of the FSC channel was checked with calibrated size beads (Calibrite Beads, Becton-Dickinson) between each blood sample studied (interassay variability 3%).

Results

Statistical analysis

SigmaStat v 3.5 software package (Systat Software Inc, San Jose, California, USA) was used. Non-parametric statistical tests were used, including the Kruskal–Wallis test and the Bonferroni test for pairwise comparisons. The correlations were evaluated by the Spearman test. Data are presented as mean (SD) or median value (25th–75th) as appropriate. A p value <0.05 was considered significant.

The multilinear regression analysis used a stepwise backward selection of the variables. The standardised regression coefficients are given for each model. The model included individual characteristics (volunteers, terminal renal failure, diabetes mellitus, sepsis or acute inflammatory state, age, RBC count, leucocyte count, platelet count, blood sugar, C reactive protein, sodium and urea concentrations and calculated osmolality). PCD and the spherical index were the dependent variables.

Results

Patients

Table 1 presents the characteristics of the subjects. The healthy volunteers were significantly younger than the patients. The causes of sepsis were pneumonia (58%), peritonitis (21%) and other causes (21%), pylonephritis, septicaemia and encephalitis). Major causes of ICU admission in patients without sepsis were postoperative inflammatory reactions (38%), intracerebral haemorrhage (14%), drug intoxication, chronic obstructive pulmonary disease and epilepsy. Of the 18 patients with diabetes, 16 had type 2 diabetes mellitus. In all, 80% of the terminal renal failure was due to nephroangiosclerosis.

Blood analyses

As expected, haemoglobin concentration and RBC count were significantly lower in critically ill patients than in patients with
diabetes and healthy volunteers. Patients with terminal renal failure were also more anaemic (despite erythropoietin treatment) than the patients with diabetes and healthy volunteers (table 2). Mean corpuscular volume was higher in patients with renal failure than in the other groups. Markers of inflammation (WBC, C reactive protein) were higher in patients in ICU. Blood sugar concentration was higher in patients in ICU and those with diabetes than in patients with terminal renal failure and healthy volunteers. Calculated osmolality was higher in patients with renal failure (owing to an increased urea concentration; table 1).

Analysis of RBC shape
The spherical index (M2:M1 ratio) was significantly lower in all patients with renal failure (owing to an increased urea concentration) than in volunteers (table 2). The PCD was significantly reduced in all diseases, corresponding to a right shift in the R1 gate (right shift of the median channel in the FSC histogram); both events reflect the spherical nature of RBCs in patients with renal failure (p = 0.002), diabetes mellitus (p = 0.002), sepsis (p < 0.001), and acute inflammatory state (p = 0.009; table 3).

Independent predictors of the PCD were terminal renal failure, diabetes mellitus, sepsis and acute inflammatory state (all p<0.001), and the RBC count (p = 0.034; table 3).

Multivariate analysis
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DISCUSSION
This rapid and easy flow cytometry technique demonstrated that RBCs in patients with diabetes, terminal renal failure, sepsis and acute inflammatory states have a more spherical shape than normal as evaluated either by the spherical index or by the PCD. These diseases are associated with alterations in RBC rheology,10–12 and these observations suggest that the deformability capacity is altered in these diseases perhaps because the RBCs are more spherical in vivo. Regrettably, only a few studies have looked at the RBC shape in these pathologies.7 10 12 21 The reason why RBCs are spherical remains unexplained and the causes are probably multifactorial (modifications of RBC membrane, reactive oxygen species and so on). Osmolality was not found to be a significant variable. In a recent study, we also observed a more spherical RBC shape in patients with sepsis than in volunteers, and these differences were not explained by different osmolalities.23 Modifications in RBC membrane composition could explain variations in RBC rigidity and alter the RBC shape.26 28 29 but these were not studied here. Further studies on haematological pathologies (membrane disorders, erythroid dysplasia in patients with myelodysplastic syndrome) are necessary to correlate the possible link between RBC protein membrane alterations and...

The spherical index (M2:M1) was strongly correlated with the PCD except for the patients without sepsis in the ICU (table 2).

Correlation between sphericity, blood sugar and HbA1C
In patients with diabetes, there was no significant correlation between the PCD or the spherical index and the blood sugar (r = −0.05; p = 0.83 and r = −0.04; p = 0.87, respectively), between the PCD and HbA1C or the spherical index and HbA1C (r = −0.24, p = 0.33 and r = −0.24; p = 0.32, respectively).

Multivariate analysis
The independent predictors of the PCD were terminal renal failure, diabetes mellitus, sepsis and acute inflammatory state (all p<0.001), and the RBC count (p = 0.034; table 3).

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Another possible use of this flow cytometry technique is the assessment of the evolution of the RBC shape after splenectomy. Using flow cytometry, we observed that the more spherical shape of the RBCs in patients with diabetes was not correlated with blood sugar or the severity of the disease as assessed by the HbA1C. These RBC alterations could increase the risk of peripheral ischaemia in these patients, especially if blood viscosity is increased. Increased adhesion of RBCs to endothelial cells in patients with diabetes was already demonstrated 20 years ago by Wautier et al and was correlated with the extent and the severity of diabetic microvascular alterations (especially nephropathy). The origin of the increased spherical shape is unclear. A possible explanation is a reduced phosphorylation of the spectrin responsible for the increased membrane viscosity in patients with diabetes. Other explanations include reduced ATP levels or an imbalance in the cholesterol:phospholipid ratio.

RBCs are also more spherical in patients with terminal renal failure requiring haemodialysis, and this alteration may contribute to the mechanical fragility of RBC membranes and to the impaired RBC deformability in these patients. A possible explanation is that patients with terminal renal failure have reduced serum antioxidant activity, and so may be more susceptible to free radical-induced injury. In our study, we could not exclude a possible effect of erythropoietin treatment on RBC shape as described by others, but it would have been unethical to discontinue this treatment.

Our study confirmed that RBCs from critically ill patients with an acute inflammatory reaction (postoperative state, nephritis, and the like) were more spherical than those of healthy volunteers. This finding is consistent with the observation that the membrane viscosity of RBCs increases in these conditions. Increased adhesion of RBCs to endothelial cells in patients with diabetes was already demonstrated 20 years ago by Wautier et al and was correlated with the extent and the severity of diabetic microvascular alterations (especially nephropathy). The origin of the increased spherical shape is unclear. A possible explanation is a reduced phosphorylation of the spectrin responsible for the increased membrane viscosity in patients with diabetes. Other explanations include reduced ATP levels or an imbalance in the cholesterol:phospholipid ratio.

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Table 2  Flow cytometry results

<table>
<thead>
<tr>
<th>RBC shape</th>
<th>PCD</th>
<th>Correlation</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septic (n = 19)</td>
<td>2.28 (0.37)*</td>
<td>-0.58 (0.22)*</td>
<td>r = -0.78, &lt;0.001</td>
</tr>
<tr>
<td>Acute inflammatory state (n = 21)</td>
<td>2.33 (0.42)*</td>
<td>-0.65 (0.29)*</td>
<td>r = -0.34, 0.13</td>
</tr>
<tr>
<td>Diabetic (n = 18)</td>
<td>2.27 (0.38)*</td>
<td>-0.59 (0.23)*</td>
<td>r = -0.78, &lt;0.001</td>
</tr>
<tr>
<td>Terminal renal failure (n = 28)</td>
<td>2.30 (0.20)*</td>
<td>-0.56 (0.14)*</td>
<td>r = -0.87, &lt;0.001</td>
</tr>
<tr>
<td>Healthy volunteers (n = 17)</td>
<td>2.72 (0.47)</td>
<td>-0.89 (0.120</td>
<td>r = -0.74, &lt;0.001</td>
</tr>
</tbody>
</table>

RBC, red blood cell.

Values are mean (SD). *(p<0.05 versus healthy volunteers.)*
intracerebral haemorrhage, chronic obstructive pulmonary disease, drug intoxication or epilepsy) were more spherical than those from healthy volunteers. These RBC alterations may contribute to impaired cellular oxygen supply. Increased RBC aggregability and decreased RBC deformability have been described in patients with sepsis. These modifications of RBC rheology probably contribute to the microcirculatory disturbances recently observed in these patients. This patient population with some inflammatory response has alterations in RBC shape and RBC membrane intermediate between patients with sepsis and volunteers.

Disease was the major factor associated with these alterations but our study cannot identify the individual factors that may be responsible in each disease state. We recommend the measurement of the PCD rather than the spherical index to estimate RBC shape in order to limit manual error.

The flow cytometry technique is a rapid technique, easier than conventional methods, and provides information about RBC shape in a large number of RBCs. This technique may be useful to better define rheological abnormalities in many disease states.

**ACKNOWLEDGEMENTS**

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**Authors’ affiliations**

M Piagnerelli, J-L Vincent, Department of Intensive Care, Erasme University Hospital, Free University of Brussels, Brussels, Belgium

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**Table 3** Multivariate analysis

<table>
<thead>
<tr>
<th>Standardised regression coefficient</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCD (n = 103), r² = 0.22</td>
<td>F = 5.3</td>
</tr>
<tr>
<td>Terminal renal failure</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Seipsis</td>
<td>0.76</td>
</tr>
<tr>
<td>Acute inflammatory state</td>
<td>0.70</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.52</td>
</tr>
<tr>
<td>RBC count</td>
<td>0.48</td>
</tr>
<tr>
<td>Acute inflammatory state</td>
<td>0.48</td>
</tr>
<tr>
<td>Spherical index (n = 103), r² = 0.13</td>
<td>F = 3.49</td>
</tr>
<tr>
<td>Seipsis</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0.41</td>
</tr>
<tr>
<td>Terminal renal failure</td>
<td>0.37</td>
</tr>
<tr>
<td>Acute inflammatory state</td>
<td>0.34</td>
</tr>
</tbody>
</table>

PCD, Pearson coefficient of dissymmetry; RBC, red blood cell.

Models included underlying pathologies, age, RBC count, leucocyte count, platelets, blood sugar, C reactive protein, sodium and urea concentrations and calculated osmolality.

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**REFERENCES**


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