Review article

Erythrocyte rheology

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SUMMARY Erythrocyte deformability was formerly measured by its contribution to whole blood viscosity. It is now more commonly measured by filtration of erythrocytes through, or aspiration into, pores of 3-5 μm diameter and by the measurement of shear induced erythrocyte elongation using laser diffractometry. Recent improvements in the technology for erythrocyte filtration have included the removal of acute phase reactants from test erythrocyte suspensions, ultrasonic cleaning and reuse of filter membranes, awareness of the importance of mean cell volume as a determinant of flow through 3 μm diameter pores, and the ability to detect subpopulations of less deformable erythrocytes. Measurements of erythrocyte elongation by laser diffractometry, using the Ektacytometer, are also influenced by cell size and need to be corrected for mean cell volume. These advances have greatly improved the sensitivity and specificity of rheological methods for measuring the deformability of erythrocytes and for investigating the mode of action of rheologically active drugs.

An earlier review of blood rheology (the science of the deformation and flow of blood) was mainly concerned with the clinical value of measuring whole blood viscosity.¹ Five years later, rheological measurements in clinical studies are more often concerned with the individual components of blood (erythrocytes, leucocytes, platelets, and plasma) that collectively influence whole blood viscosity. It is therefore appropriate to review the residual role of measurements of whole blood viscosity while concentrating on new developments in the study of erythrocyte rheology.

Measurement of whole blood viscosity

This measurement still has a role as a global test, but its relatively poor sensitivity and specificity, and overdependence on the packed cell volume, have limited its clinical usefulness. Within the normal range there is a linear relation between packed cell volume and the logarithm of whole blood viscosity, but attempts to correct viscosity to a standard packed cell volume of 0.45, using regression lines, are prone to error.² Regression lines derived from normal blood may not apply to patients' samples,

and it is often impractical to derive a personalised regression line from each patient's blood sample. Mathematical formulas can be used to correct viscosity to a standard packed cell volume,³ but it is easier to reconstitute blood samples to a standard erythrocyte count in autologous plasma or buffer and then measure erythrocyte viscosity.

At low shear rates ($<1/\text{s}$) measurement of erythrocyte viscosity in autologous plasma will reflect the formation of erythrocyte rouleaux, which is proportional to the plasma concentration of large molecular weight proteins with a high frictional ratio, such as fibrinogen.⁴ Part of the rheological effect of plasma proteins can, however, be measured more simply as plasma viscosity. At high shear rates ($>100/\text{s}$) measurements of viscosity will reflect erythrocyte deformability as cell-cell interactions are dispersed and the originally discocytic erythrocytes become deformed into elliptocytes that rotate (tank tread) their membranes.⁵ Other rheological methods, however, are probably more sensitive to loss of deformability,⁶ so that the role of high shear viscometry is again limited.

The development of new rheometers with the potential to measure whole blood elasticity and the cell-cell interaction caused by plasma proteins may generate new applications for whole blood systems,

but the current trend is very much towards the rheological study of individual blood components.

Measurement of erythrocyte deformability

Human erythrocytes, when subjected to extrinsic shear stress in the circulation, are able to deform because of their low cytoplasmic viscosity, high ratio of surface area to cell volume, and viscoelastic membrane. Such deformation includes their linear alignment as ellipsoids, with rotation of their membranes, during arterial flow⁷ and also folding of the cells to produce "slipper" and other forms in the microcirculation.⁸ The principles of deformability and the wide variety of available methods for its measurement have recently been reviewed.^{9,10} Of these methods, only two (filtration of erythrocytes through pores of 3–5 μm diameter and the measurement of erythrocyte elongation using laser diffractometry) have been widely applied clinically. As a combination of the two approaches provides a useful rheological profile for clinical studies⁶ this review will concentrate on these measurements of erythrocyte filtration and elongation.

ERYTHROCYTE FILTRATION

Measurement of erythrocyte flow through the 3–5 μm diameter pores of polycarbonate membranes (Figs. 1 and 2) gained considerable clinical impetus after the development of a simplified filtration apparatus.¹¹ This method of whole blood filtration was subsequently modified¹² because of its original high shear stress and susceptibility to the effects of variation in packed cell volume, aggregation of red cells, and contamination with leucocytes.¹³ Others had shown that the flow of erythrocytes through 5 μm pores was influenced by contaminating leucocytes at counts above, within, and below the physiological range,^{14–16} and the new method eliminated at least 75% of leucocytes.¹³ Subsequent workers, however, showed the dominating effect on filtration of virtually any residual leucocytes in such systems, whether of gravity filtration^{17,18} or positive pressure^{19–21} type. In gravity flow systems contaminating leucocytes should be reduced to $<0.025 \times 10^9/\text{l}$.¹⁸

A simple method for the rapid (15 minutes) removal of contaminating leucocytes to $<0.025 \times 10^9/\text{l}$ from small volumes of blood is the prefiltration of 2 ml, or more, of anticoagulated blood through Imugard IG500 (Terumo Corporation, Tokyo, Japan) *Gossypium barbadense* cotton wool.^{18,19} Centrifugation (1850 g for 10 minutes) in narrow bore (3 mm diameter) Dade Wintrobe macrohaematocrit tubes of 1.0 ml capacity²² or in wider bore tubes²³ may also remove sufficient leucocytes, but there is

Fig. 1 Scanning electron photomicrograph of top surface of 3 μm pore diameter Nuclepore polycarbonate membrane showing pore coincidence $\times 1500$.

often inadequate separation of the buffy coat layer in anaemic subjects.¹⁸ Other methods of removing leucocytes, using phthalate ester density gradients,^{24,25} microcrystalline cellulose,²⁶ or dextran and isopaque,¹⁹ are either too lengthy or remove insufficient leucocytes. High gradient magnetic separation of erythrocytes is a different approach of potential value.^{27,28}

It is not entirely clear why residual leucocytes must be reduced to such a low number as it has been calculated that only one pore in 10 of a 5 μm Nuclepore polycarbonate membrane would be traversed

Fig. 2 Scanning electron photomicrograph of section of 5 μm pore diameter Nuclepore polycarbonate membrane showing straight channel pores $\times 4500$.

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by a leucocyte if no reduction of leucocytes were attempted before erythrocyte filtration in a Hemorheometre.²⁹ In some filtration systems, however, disruption of leucocytes may cause the lower (exit) surface of polycarbonate membranes to become coated with a fine meshwork that can cause erythrocytes to be entrapped in the form of a bouquet.^{30,31} Whatever the explanation, the effect of leucocytes on the filtration of erythrocytes is likely to be more than simple plugging of the pores.

Additional components in whole blood with the potential to impair the filtration of erythrocytes are the larger plasma proteins such as fibrinogen, which cause aggregation of erythrocytes in the filter holder,^{14,32} and platelets, which can obstruct filter pores when microaggregated by heparin anticoagulants.^{17,33} The Imugard IG500 prefiltration technique, which is followed by washing the erythrocytes in buffer, removes >99% of platelets and most of the plasma protein.

Advantages of testing a pure erythrocyte suspension

When whole blood is filtered it is difficult to attribute a rheological abnormality to any one particular component. When a pure erythrocyte suspension is filtered, however, the sensitivity and specificity of the technique for an abnormality of erythrocyte deformability is enhanced. Moreover, the patients' blood specimens can be anticoagulated with either heparin or edetic acid and then kept at room temperature for up to six hours before testing.¹⁷ Most importantly, the results obtained are independent of any acute phase increase in blood leucocyte count or fibrinogen concentration.

Acutely ill patients invariably show an acute phase response, mediated via interleukin-1,^{34,35} with release into the blood within three to six hours of mature granulocytes from the bone marrow^{36,37}; this is followed, at about six hours after the onset of the illness, by an increase in plasma fibrinogen concentration.^{37,38} As both leucocytes and fibrinogen have the potential to impair the filtration of erythrocytes earlier reports of a loss of erythrocyte deformability in the acute phase of myocardial infarction,³⁹ stroke,⁴⁰ and surgery⁴¹ should be reinterpreted.

Chronic disorders also produce a stress response,^{42,43} and the impaired filterability of erythrocyte suspensions contaminated with leucocytes in peripheral occlusive arterial disease,¹⁵ diabetes mellitus,⁴⁴ and pre-eclampsia⁴⁵ has been shown to reflect the slight leucocytosis of these conditions. Other rheological aspects of the acute and chronic phase responses have been reviewed elsewhere.^{4,46}

Removal of fibrinogen and, in particular, leucocytes from rheological test suspensions is therefore

required when using current filtration systems; this also facilitates the matching of patients with controls. Increasing age causes a rise in plasma fibrinogen concentration,⁴⁷ and smokers⁴⁸ and stressed patients may show a leucocytosis. If acute phase reactants are not removed from test erythrocyte suspensions then the above factors must be carefully matched in the control group.

Disadvantages of preparing a pure erythrocyte suspension

Any postvenepuncture manipulation of the erythrocyte entailing removal from its normal environment of plasma has the potential to alter erythrocyte rheology. Washing in buffer, for example, removes surface protein from the erythrocyte and may remove membrane lipid⁴⁹; the filtration of washed erythrocytes suspended in Locke-albumin buffer has been found to improve once fibrinogen and cholesterol are added to the buffer.⁵⁰ Furthermore, suspension in Tris buffer increases the mean cell volume of erythrocytes⁵¹ and causes rapid impairment of erythrocyte filterability.⁵² As filtration through 3 μ m diameter pores is particularly sensitive to mean cell volume the buffer pH and osmolality must be carefully controlled to avoid swelling of erythrocytes.

In all rheological studies the original *in vivo* environment of the erythrocyte should be remembered when *in vitro* measurements are made. At an ischaemic site erythrocytes are likely to be exposed to low oxygen tension, low pH, and raised osmolality. This will no longer apply when the cell reaches the site of venepuncture in the cubital fossa and will certainly not apply when the cell is suspended in buffer *in vitro*. Thus it may be necessary to simulate *in vitro* the metabolic environment experienced by the erythrocyte *in vivo*.⁵³

Finally, all methods of removing leucocytes have the inherent risk that any subpopulation of less deformable erythrocytes may be either selectively trapped in cotton wool prefilters or separated according to their density in centrifugation techniques. Ideally, filtration methods that are independent of the effects of leucocytes, platelets, and proteins should be developed, so that whole blood can be filtered. For existing instruments the real advantages of preparing a pure, albeit washed, erythrocyte suspension outweigh the theoretical disadvantages; as more sensitive rheological techniques are developed, however, the artificial nature of studying washed erythrocytes should not be forgotten.

Which type of membrane to use?

Since their introduction in 1967⁵⁴ polycarbonate membranes (Figs. 1 and 2) with straight channel

pores of 5 μm diameter and 10–11 μm length (Nuclepore Corporation, Pleasanton, California, United States) have been used. They have the advantages of being disposable and easy to handle but are expensive, and the interbatch production quality control is poor. It has become customary, following the recommendations of the Royal Society of Medicine Working Group report on red cell deformability in 1981,⁵⁵ to filter buffer first through each membrane and then express the filtration result for erythrocytes suspended in that buffer in relation to buffer alone. Although helpful, this does not compensate sufficiently for variation between batches. Not only do membrane batches differ in their pore diameter but the stated diameter may differ from the measured value.³¹ Four batches of stated 5 μm pore diameter were found, in practice, to contain membranes with pores ranging from 1 to 6 μm in diameter.⁵⁶ A considerable number of confluent pores (Fig. 1) are found in most batches, and these influence the flow rate and exacerbate the shunting of erythrocytes between pores.⁵⁷

Although disposable, individual polycarbonate membranes can be used repeatedly by ultrasonication at 58 kHz for 10 seconds in 1% w/v aqueous sodium dodecyl sulphate between each filtration.⁵⁸ This substantially improves the precision of measurement as the same membrane can be used for both test and control samples, and it also reduces the cost.

Straight channel, reusable metal membranes have the potential for better quality control during production and extended life span; commercial production has recently started (MyNiPore metal membranes, Myrenne GmbH, D-5106 Roetgen/Aachen,

West Germany). Reusable sintered silver membranes (Osmonics Inc, Minnetonka, Minnesota, United States) with tortuous channels (Fig. 3) have also been used^{6,50,59,60} and performed similarly to polycarbonate membranes of similar (5 μm) pore diameter in a clinical study.⁶ Despite the conceptual attraction of these long (≈ 50 –70 μm) and tortuous branching channels, which simulate the microcirculation, there are practical advantages in using straight channel pores. The shear stress at the wall can be calculated and the physical behaviour of an erythrocyte within the channel more readily predicted. The interior channels of a sintered silver membrane are continuously variable, and the repeated ultrasonication required for cleaning may cause internal and, therefore, unseen disintegration. A quality assurance programme, using the flow time for dextran 70,⁵⁹ for example, is therefore essential. Silver membranes are, however, less sensitive to the effects of contamination with leucocytes⁵⁹ and to mean cell volume,⁶¹ so that further evaluation of silver membranes compared with straight channel metal membranes will be of value.

Which pore size to use?

Flow of erythrocytes through narrow (3–5 μm diameter) channels of 10 μm length is governed by the three main determinants of erythrocyte deformability—namely cell geometry (including the ratio of surface area to volume); membrane elasticity; and cytoplasmic viscosity. The relative contribution of these determinants to flow will, however, differ according to the diameter of the pore.

Flow of erythrocytes through 5 μm diameter

Fig. 3 Scanning electron photomicrograph of top surface of 5 μm (nominal) diameter tortuous channel sintered silver membrane (Osmonics Inc) $\times 1500$.

Fig. 4 Effect of alteration in osmolality on erythrocyte mean cell volume and mean cell haemoglobin concentration and Hemorheometre index of filtration using 3 μm (●) and 5 μm (○) diameter straight channel polycarbonate membranes. Index of filtration at physiological osmolality (290 mmol (mosmol)/kg) has been expressed as 100%.

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pores is sensitive to mean cell volume when either above^{62,63} or below⁶⁴ the normal range, but within the normal range the correlation between flow and mean cell volume is weak.^{6,60} As shown in Fig. 4, an increase in mean cell volume from 85.5 to 92 fl, induced by suspension of erythrocytes in hypotonic buffer, had little effect on the flow of erythrocytes through a 5 μ m pore diameter polycarbonate membrane, using a Hemorheometer. The index of filtration increased from 100% to 330%, however, when the erythrocytes were filtered through a polycarbonate membrane of 3 μ m pore diameter. Conversely, when hypertonic buffer was used to increase erythrocyte mean cell haemoglobin concentration progressively from 34.8 to 39.1 g/l (Fig. 4) filtration of erythrocytes through 5 μ m diameter pores reflected the resulting increase in cytoplasmic viscosity, more so than flow through 3 μ m pores.⁶¹ Presumably, erythrocyte geometry has such a dominant effect on the entry of cells into, or flow of cells through, 3 μ m pores that sensitivity to any increase in mean cell haemoglobin concentration is reduced. Erythrocytes already fit tightly as they pass through 5 μ m diameter pores⁶⁵ and as pore diameter decreases towards the critical value for cell passage changes in intracellular viscosity become less important than cell size.⁶⁶

Only a few studies on filtration have selected membranes of 3 μ m pore diameter^{50,67-74} and even fewer have included both 3 μ m and 5 μ m pore membranes.^{31,60,64,75,76} In one of these studies⁷⁵ an effect of prostacyclin on the filterability of erythrocytes was detected using 3 μ m, but not 5 μ m, pores. As evidence shows that 3 μ m pores may be more sensitive than 5 μ m pores to minor changes in the deformability of erythrocytes³¹ this effect of mean cell volume will become increasingly important in the current trend towards the use of smaller diameter pores.

An increase in mean cell volume also affects the Single Erythrocyte Rigidometer,⁵⁶ aspiration of erythrocytes into 3 μ m diameter micropipettes,⁷⁷ and elongation of erythrocytes in the Ektacytometer.^{6,60,78-80} In view of the sensitivity of these methods, including 3 μ m pore filtration to mean cell volume, great care should be taken when new batches of buffer are prepared. Any reduction in tonicity or in the pH, which also increases mean cell volume,⁸¹ will affect the results; osmolality, in particular, should always be checked by the freezing point depression method once the working buffer has been prepared.

In clinical studies it may be necessary to use both 3 μ m and 5 μ m diameter pores, depending on the disorder to be studied and the expected changes in mean cell volume and mean cell haemoglobin concentration. Polycarbonate membranes with

intermediate (≈ 4 μ m) diameter pores are not available but would be of considerable interest for comparative studies. Needless to say, mean cell volume and mean cell haemoglobin concentration should be recorded in all rheological studies, so that the results can be interpreted in relation to these important determinants of erythrocyte rheology.⁸² Calculated values of mean cell haemoglobin concentration should not be based on a centrifuged packed cell volume, however, as reduced deformability causes an exponential increase in trapped plasma, giving a spuriously high packed cell volume and thus underestimated mean cell haemoglobin concentration.⁸³

Which filtration instrument to use?

Such has been the rate of development of rheological instruments in recent years that new instruments for filtration have appeared faster than existing ones can be evaluated. Currently available commercial instruments have recently been described¹⁰ but few comparative studies have been completed.^{6,84-87} This reflects the difficulty of amassing sufficient instruments in one centre plus the problem of standardising the numerous technical variables such as membrane composition and pore size, filtration pressure (positive, negative, or gravity), contamination with leucocytes and temperature of measurement.

Studies to evaluate instruments should, preferably, include samples of patients' blood as well as in vitro manipulated erythrocytes, as the results may not correspond.⁶ Positive pressure (constant flow) systems, for example, have been extensively used^{20,30,32,67,71,88,89} and have performed well in in vitro studies.⁸⁵ Their precision, however, depends on the sensitivity of the pressure transducer and the smoothness of the pump. Thus testing of replicate specimens has given the relatively poor coefficients of variation of 10-12% for a self made system⁸⁵ and $\approx 7\%$ for a commercially made instrument when 5 μ m pore membranes were used.^{6,89} In clinical studies a high coefficient of variation gives a relatively wide reference range for the control group, and this has reduced the ability of a positive pressure system to differentiate normal from abnormal specimens.⁶

Gravity flow filtration systems are simpler to construct, and lower coefficients of variation ($<3\%$) have been obtained.⁶ This has given better differentiation than positive pressure filtration between control and patient groups in a study of arterial disease, renal disease, liver disease, and excess alcohol consumption.⁶

Commercial examples of gravity flow (initial flow rate) instruments include the Filtrometer MF4 (Myrenne GmbH, D-5106 Roetgen/Aachen, West

Fig. 5 Sectional drawing of Hemorheometre showing water jacket, two optical flow detectors, and horizontal location of filter membrane.

Germany),⁹⁰ which has been previously described¹⁰ and evaluated⁸⁴⁻⁸⁶; the Hemorheometre Mk 1 (IMH, 95470 St Witz, France)⁹¹; and the St George's Blood Filtrometer (Carri-Med Ltd, Dorking, Surrey).⁸⁷ Various membrane filters of 3–5 μm pore diameter may be used in each of these instruments. One major difference lies in the facility for controlling temperature in the Hemorheometre and St George's Blood Filtrometer: this is important in laboratories that do not have a constant ambient temperature (see below). A second major difference is the number of flow detectors that measure the rate of filtration. This ranges from two in the Hemorheometre to four in the St George's Blood Filtrometer (Fig. 6) while continuous monitoring is available in the Filtrometer. Multiple flow detectors allow more sophisticated analysis of the flow rate with differentiation between initial flow (transit time) through the membrane and subsequent clogging of the pores. The initial flow is less dependent on contamination with leucocytes.⁸⁷ As yet, however, there has been no comparative study of these three instruments, and their respective advantages remain to be proved in clinical studies.

Fig. 6 Sectional drawing of St George's Blood Filtrometer showing four flow detectors and vertical location of filter membrane.

Other technical variables in filtration studies

It has been recommended⁹⁵ that a dilute cell suspension, within the range of packed cell volume 0.01–0.1, should be used for studies on filtration as resistance to flow is less dependent on packed cell volume at these low levels. This range is probably too wide for some correction formulas for packed cell volume, such as those recommended for the Hemorheometre,⁹¹ and the adequacy of the correction should be checked if packed cell volumes of <0.05 are to be used. It is also difficult to measure the packed cell volume accurately at these low levels.

Measurements of filtration have usually been made at ambient temperature, but variation in laboratory temperature within the range of 20–25°C has been shown to influence filtration of erythrocytes through 5 μm diameter pores in the Hemorheometre.⁹² Others have found that the filtration of erythrocytes through 5 μm pores depends on temperature within the range 32–42°C.⁹³ Dependency of temperature is, however, affected by the size of pore, the reduction in the filterability of erythrocytes at 20°C compared with 25°C being greater for 3 μm than for 5 μm diameter pores.⁶¹ Thus instruments for filtration should be controlled for temperature. There is no agreement on the optimal working temperature, but the upper level of the ambient temperature range (25°C) has practical advantages as it is more technically demanding to maintain buffer, erythrocyte suspension, syringe, flow system, and filter membrane at higher temperatures such as 37°C. As flow times may be very rapid (<0.5 seconds) the sample should already be at working temperature when introduced into the instrument.

The temperature at which whole blood should be stored between the time of venepuncture and that of testing is also important. Storage of whole blood at 37°C for longer than 20 minutes has been associated with a significant increase in the whole blood filtration time.⁹⁴ Storage of whole blood at 4°C for six hours may result in loss of erythrocyte potassium and a gain in sodium.⁹⁵ As whole blood has been stored at ambient temperature for up to six hours without an appreciable change in 5 μm pore filterability of a subsequently prepared pure erythrocyte suspension¹⁷ there would not seem to be any advantage in storage at either higher or lower temperatures.

The shape of the erythrocyte is another determinant of deformability,⁹⁶⁻⁹⁷ and a change in shape induced artificially by, for example, contact with glass⁹⁸ or an inappropriate buffer should be avoided. Positive pressure filtration through narrow (2.6 μm) diameter pores has also been reported to cause cre-

Fig. 7 Sectional drawing of Ektacytometer showing helium-neon laser beam passing through erythrocyte test suspension to generate elliptical diffraction image as erythrocytes are sheared by rotation of inner cylinder.

nation of erythrocytes,³¹ presumably secondary to subhaemolytic damage, and filtration systems causing high shear stresses (>100 Pa) within the membrane pores should be avoided. In all studies it is of value to monitor the morphology of test cell suspensions regularly.

MEASUREMENT OF ERYTHROCYTE ELONGATION

The Ektacytometer (Technicon International Division, St Denis, France)⁹⁹ combines viscometry with laser diffractometry. It consists of a cylindrical Couette viscometer which is translucent, thus allowing a helium-neon laser beam to pass through the erythrocyte test suspension during rotational shear (Fig. 7). Test erythrocytes are suspended at a packed cell volume of ≈ 0.002 in 3.1% w/v polyvinylpyrrolidone and subjected to a shear stress of up to 16 Pa at ambient temperature. By altering the sodium chloride content of the buffer the osmolality of the suspending medium can be progressively varied from 100 to 450 mmol (mosmol)/kg, so that

the test erythrocytes are exposed to osmotic stress during rotational shear. The laser diffraction image (Fig. 7) becomes elliptical as the erythrocytes are sheared, and the ratio of the major and minor axes of the image is designated the elongation index. As the system lends itself to flow analysis the progressive increase in osmolality allows the elongation index to be determined under hypotonic, isotonic, and then hypertonic conditions with the serial results displayed on an x-y recorder as an Osmoscan plot (Fig. 8). Four Osmoscan variables are usually measured—namely, EI max, or the maximum value of the elongation index obtained (usually at isotonic osmolality); O max, or the osmolality at which EI max occurs; O min, or the osmolality at which the minimum value for the elongation index is obtained in the hypotonic arm of the curve; O hyper, or the osmolality at which the elongation index decreases to half the value obtained for EI max in the hypertonic arm of the curve.

This dynamic measurement of erythrocyte elongation has been used mainly for the rheological study of congenital defects of erythrocyte membrane protein, as in elliptocytosis,¹⁰⁰ ovalocytosis,¹⁰¹ and hereditary spherocytosis.¹⁰² The Osmoscan plot is also sensitive to other pathological changes, including alteration in the shape of erythrocytes,^{97 103} loss of cell water,¹⁰⁴ polymerisation of sickle haemoglobin,¹⁰⁵ and coating with antibody.¹⁰⁶ Membrane properties of resealed erythrocyte ghosts can also be studied.¹⁰⁷

Any reduction in the normally favourable ratio of cell surface area to volume (as in autoimmune haemolytic anaemia with spherocyte formation secondary to membrane loss) will cause the hypotonic arm of the Osmoscan to move to the right (Fig. 9). Conversely, the excess membrane area of the codo-

Fig. 8 Ektacytometer Osmoscan showing effect of osmolality variation on erythrocyte elongation index.

Fig. 9 Ektacytometer Osmoscan for normal subject and right shifted Osmoscan for patient with autoimmune haemolytic anaemia and spherocyte formation. Vertical interrupted lines show osmolality shift at midpoint of hypotonic arm of curve.

cytes (target cells) of liver failure will cause the hypotonic arm to move to the left (Fig. 10). At physiological osmolality, EI max is reduced as the elasticity of the membrane decreases.

Few Ektacytometric studies in rheological disorders other than the haemolytic anaemias have been made.^{6 60 97 108 109} In disorders associated with an in vivo abnormality of the shape of erythrocytes (for example, the codocytes of liver failure⁹⁷) or when echinocytes and spheromatocytes have been induced in vitro⁶ the Osmoscan will usually detect the abnormality. Values for the elongation index are particularly sensitive to loss of elasticity of the membrane, as was also shown with a rheoscope ektacytometer.⁸⁴ Thus damage to the membrane caused by heating to 47.5°C or by exposure to low (0.001–0.003% w/v) concentrations of glutaraldehyde is more readily detected by a reduction in EI max than by filtration through 5 µm diameter pores.⁶ Erythrocyte mean cell volume, however, has a much greater effect on EI max, which is not com-

Fig. 10 Ektacytometer Osmoscan for normal subject and left shifted Osmoscan for patient with liver failure and codocytes (target cells). Vertical interrupted lines show osmolality shifts at midpoints of hypotonic and hypertonic arms of curve.

pensated for cell size, than on filtration through large (5 µm) diameter pores.^{6 60} Figure 11 shows this effect of mean cell volume, and a significant correlation ($r = 0.574$, $p < 0.001$) between EI max and mean cell volume has been obtained in 58 healthy controls.⁸⁰ As mean cell volume is often abnormal in patients with an acquired rheological disorder⁶ it is necessary to correct EI max values for mean cell volume using, for example, a regression line.

The Ektacytometer is a sophisticated and versatile instrument with the advantages of a small (100 µl) sample volume, low running costs, and a particularly low coefficient of variation ($\approx 1\%$) for replicate estimations. The low coefficient of variation increases the sensitivity of the Osmoscan to small changes in the elasticity of the erythrocyte membrane, the geometry and shape of erythrocytes, and the cytoplasmic viscosity. Disadvantages of the instrument include its high initial cost and the sensitivity of EI max to mean cell volume.

STUDY OF SUB POPULATIONS OF ERYTHROCYTES Although the Ektacytometer has been claimed to detect small populations of undeformable cells,⁹⁹ neither this instrument nor any of the instruments for bulk filtration described above is suited to the study of subpopulations. This is an important disadvantage owing to the rheological heterogeneity of normal and pathological erythrocytes.

As the normal erythrocyte approaches the end of its life span in vivo it loses membrane area and volume^{110 111} and its mean cell haemoglobin concentration increases secondary to loss of potassium and, therefore, water¹¹²; the resulting increase in density allows older erythrocytes to be separated by centrifugation. These denser cells show impaired filtration through both 5 µm¹¹³ and 2.6 µm³¹ diameter pores and require a greater aspiration pressure to enter micropipettes of 2.8 µm diameter or less.¹¹⁴ It is unknown whether the rheological heterogeneity of erythrocytes from normal subjects becomes exaggerated in patients with a rheological disorder. In diabetes mellitus, for example, the oldest (most dense) erythrocytes have shown, compared with younger cells, abnormal deformability in a cone plate rheoscope¹¹⁵ (but not any more so than aged non-diabetic erythrocytes¹¹⁶); impaired filtration was also shown in a Filtrometer MF4.¹¹⁷ Heterogeneous erythrocyte populations are also known to exist in sickle cell anaemia,¹¹⁸ in spherocytosis,¹¹⁹ and in the neonate.¹²⁰

It is therefore important to establish the degree of rheological heterogeneity in other disorders as a subpopulation of 5–10% of less deformable erythrocytes may be sufficiently diluted by normal erythrocytes in whole blood to be undetectable by cur-

Fig. 11 Ektacytometer Osmoscan plots showing maximal erythrocyte elongation (EI max) as function of mean cell volume in three patients with macrocytosis, normocytosis, and microcytosis, respectively.

rent rheological instruments. The simplest approach to this problem is to separate, by centrifugation or a density gradient, the young and old erythrocyte fractions and test them rheologically, using currently available bulk flow instruments. Removal of leucocytes is again important if filtration instruments are to be used.

Individual erythrocytes can also be studied by micropipette aspiration or in the Single Erythrocyte Rigidometer. These alternative approaches may be preferable as the less deformable erythrocytes are not necessarily the most dense cells.

Micropipette aspiration

Glass micropipettes of 1 μm or 3–4 μm in diameter can be used to aspirate part of the membrane or all of an erythrocyte, and the aspiration pressure can then be measured (reviewed by Stuart *et al*).¹⁰ Micropipettes with a straight channel or a constricted channel can be used. The large analytical variability of the technique is likely to be improved by measuring at standard temperature and by semiautomating the production of micropipettes.¹²¹ Despite these potential improvements the population of cells that can be sampled by this technically demanding procedure is likely to remain so small as to limit the detection of subpopulations.

Single Erythrocyte Rigidometer

The time required for 200 individual erythrocytes to pass through a single pore of 3–6 μm diameter can be measured using the Single Erythrocyte Rigidometer (Hoechst AG, Frankfurt, West Germany). Passage through the pore, located in a plastic (Macrofol N) membrane,¹²² is measured by change

in electrical resistance.¹²³ As in techniques for filtration and micropipette aspiration the pore diameter will be a critical factor in determining erythrocyte deformability, narrower pores being sensitive to any increase in mean cell volume.⁵⁶ If this instrument, which is shortly to become commercially available, is to be used for the study of erythrocyte subpopulations then more than 200 cells may need to be sampled.

Evaluation of prototypes of the instrument has shown promising sensitivity, in comparison with bulk flow instruments, for in vitro manipulated erythrocytes⁸⁴ and for the detection of the effects of drugs on erythrocytes.⁸⁶ Specificity of the instrument for changes in erythrocyte deformability, as opposed to the effects of leucocytes and plasma proteins, will need to be determined.

Cell Transit Time Analyser

A new instrument, in which the flow of 1000 cells through 10–20 pores (5 μm diameter) in a masked Nuclepore polycarbonate membrane is measured, has recently been described.¹²⁴ This Cell Transit Time Analyser measures change in electrical conductance when a pore is occupied by a single erythrocyte, the time of passage being longer for less deformable erythrocytes. The transit times are then displayed as a histogram. No evaluation of the instrument is yet available.

COMBINATIONS OF INSTRUMENTS

The availability and relatively low cost of filtration instruments have been the reasons for their widespread application to clinical studies. Results obtained using 5 μm pores have shown that filtration

methods are primarily sensitive to cytoplasmic changes such as an increase in mean cell haemoglobin concentration (Fig. 5) or the formation of Heinz bodies.^{6,84} This also applies to filtration through larger diameter pores (6–8 μm) as erythrocytes swollen in hypo-osmolar buffer, and therefore showing a decreased mean cell haemoglobin concentration, may flow faster than normal sized cells.⁸⁴ Only when the pore diameter is decreased towards 3 μm in diameter does cell size exert a dominant effect over cytoplasmic viscosity (Fig. 4). Although a combination of pore sizes (for example 3 and 5 μm) is therefore recommended to increase the rheological information obtained from filtration methods, these methods remain intrinsically less sensitive to damage to the erythrocyte membrane and alteration in shape compared with the Ektacytometer Osmoscan.⁶

The combination of a gravity filtration system (using two pore sizes) and the Ektacytometer Osmoscan (with correction of elongation index for mean cell volume) would therefore seem to offer a wide rheological profile that is sensitive to the main determinants of erythrocyte deformability. It will be interesting to determine whether any single new instrument, such as the Single Erythrocyte Rigidometer, can match the wide range of this rheological profile in clinical studies.

Conclusions

Rheological methods have advanced rapidly in the past five years and there is now a clear historical progression from the earlier measurements of whole blood viscosity and whole blood filtration to the current selective study of leucocyte rheology,^{125–126} platelet rheology,^{127–129} and erythrocyte rheology. Progress in erythrocyte rheology has included realisation of the need to remove contaminants extrinsic to the erythrocyte in order to improve the specificity of filtration methods. A significant increase in sensitivity is now likely to be achieved by ultrasonication and reusing filter membranes, using a combination of different membranes and instruments, and investigating erythrocyte subpopulations. These advances in specificity and sensitivity, allied to improvements in the design of both clinical and laboratory studies,¹³⁰ should lead to better understanding of the rheological basis of vascular disorders.

Finally, it must be remembered that erythrocytes do not exert their rheological effect in isolation. Size and deformability of erythrocytes^{131–132} influence adherence of platelets to arterial subendothelium, and sublytic erythrocyte injury induced by shearing at sites of atherosclerotic stenotic lesions may liber-

ate sufficient adenosine diphosphate to potentiate aggregation of platelets.¹³³ Erythrocytes are themselves influenced at low shear by the plasma fibrinogen concentration, which is an important risk factor for vascular thrombotic events.^{134–136} Rheological studies of the interaction of erythrocytes with other blood components are clearly important in vascular disorders, such as diabetes mellitus,¹³⁷ and are likely to be a particularly fertile research area.

I am indebted to Mr JA Dormandy for providing Fig. 6.

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