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Size- and density-dependent elution of normal and pathological red blood cells by gravitational field-flow fractionation☆

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Abstract

Elution of normal and pathological human red blood cells (RBCs) was performed by gravitational field-flow fractionation (GFFF). The reproducibility of the retention factor was lower than 10% and elution at high and low flow-rates confirmed the existence of "lifting forces". No direct correlation between size and retention was observed for normal RBCs in the absence of density information. Elution of pathological human RBCs, known to be modified in shape, density and rigidity, was performed. The elution parameters confirmed that the retention mechanism of RBCs is at least density dependent but that other factors can be involved, such as shape or deformity. Moreover, peak profile description parameters (standard deviation and asymmetry) can be qualitatively related to some biophysical parameters. Numerous elution characteristics can be linked to cell properties described in the literature and although GFFF appeared to have limited capabilities in terms of size analysis it appeared to be a versatile tool for studying cell biophysical characteristics.

1. Introduction

In haematology, red blood cell (RBC) analysis is systematically performed for measurement purposes, part of the objectives being the sizing and evaluation of the size dispersion. Cell counters used in haematology usually give different parameters concerning RBCs: number, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). However, evaluation of the dispersion of RBC volumes is generally reduced to an index (RDW) corresponding to the width of the distribution curve of RBC volumes. The density and density distribution of RBCs are not described in the classical panel of clinical analysis. However field-flow fractionation (FFF) subtechniques give access to the analysis of the size distribution of normal RBCs from different species [1].

We have therefore investigated the elution of RBCs in gravitational FFF (GFFF), using normal RBC populations of different MCV, to analyse the possible correlation of size with the

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retention of the particles. RBCs from patients suffering from either β-thalassaemia [2] or sickle cell disease [3] with abnormal volumes, densities, shapes and deformability were also studied.

Elution of RBCs, like latex particles, in GFFF appeared to be flow dependent, and their elution mechanisms have been described [4,5]. As shown schematically in Fig. 1 and depending on the flow velocity, on the channel thickness and on the field intensity, the elution of micrometre-sized particles is driven by at least a balance of their size and density, the relative importance of which is still difficult to formalize. Size-driven separations can be achieved at low flow-rates, under high external field, and this elution mode is described as "steric". When flow-rates increased at constant external field (in a channel of given thickness), inertial or lubrication forces [6,7] acting perpendicularly to the particle displacement axis compete in a complex manner with the external field and modify retention. Consequently, focusing of the particles in the channel thickness occurs and this elution mode is usually described as "hyperlayer". This is why this elution mode describes one potential use of FFF, *i.e.*, in a possible density analysis of RBCs. The density distribution and rigidity of sickle cells are modified in comparison with normal RBCs; thalassaemia cells appear to be of lower density because of a decreased haemoglobin concentration. They are also more spheric than normal cells.

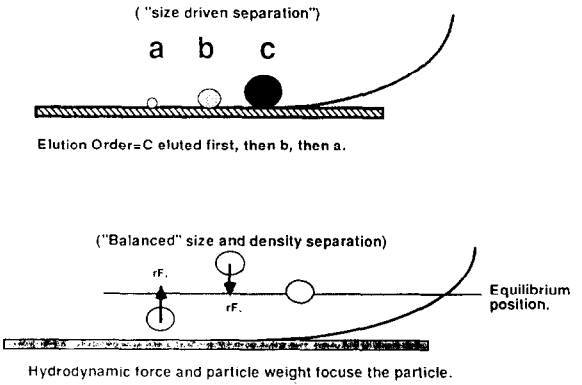

Fig. 1. Elution principle of micrometre-sized particles in an FFF channel. (Top) Steric elution mode: size selectivity. (Bottom) Hyperlayer elution mode: size and density selectivity described by the model. rF = force resulting from the mass and hydrodynamic force balance.

2. Experimental

2.1. Field-flow fractionation

The separation channel used in this work has been already described [8–10]. Two mirror-quality pieces of glass ($1 \times 10 \times 100$ cm), previously drilled for tubing, were coated according to the following procedure: the glass plates were immersed in sulphochromic acid solution, then washed under a constant flow of doubly distilled water until the pH of the washings was >6.5. The glass plates where then gently dried in a thermostated dust-free box. Silbione emulsion from Rhône-Poulenc-Rorer (Paris, France) was diluted tenfold with doubly distilled water and the glass plates were immersed in the emulsion for 24 h. After gentle cleaning with buck skin, the glass plates were heated at 150°C for 12 h. For experiments on the sizing of RBC in GFFF, a 250-μm thick channel was chosen for comparison with the thickness mostly used in FFF.

The carrier phase used for all experiments was an isotonic phosphate buffer of pH 7.4. A 50-μl aliquot of the RBC suspension dilution was injected into the channel inlet through a septum device in the absence of flow, the flow being resumed after 5 min (injection with relaxation). The cell detection system at the outlet of the channels was a classical Tridet chromatographic spectrophotometer from Perkin-Elmer (Paris, France) operated at 254 nm, or a Knauer variable-wavelength monitor (Cunow, Cergy, France) operated at 313 nm.

2.2. Red blood cells

All the human RBCs used were drawn from a patient on EDTA K3. Sample dilutions were analysed, in the following 4h, with a Minos ST counter device (ABX, Montpellier, France) to determine the average volume and red cell volume distribution. In the following studies

performed on normal and pathological RBCs the MCV is given and a normal RBC of the same average volume is systematically used as reference for comparisons. As it was not possible to find thalassaemia cells and normal cells of the same average volume, the size difference was deduced from the measured average volumes. The experiments were performed in the same silicone-coated channel, and particular attention was paid to elute the RBCs under reproducible hydrodynamic conditions. Systematic dilution of the blood suspension in an isotonic phosphate buffer (pH 7.2) was performed to obtain fairly constant injected cell numbers (*ca.* 10^6 cells were injected in 50 μl).

2.3. Peak analysis

Fraction collections were performed to check the elution of RBCs. Sizing was performed before and after experiments with a Minos ST cell counter. In FFF experiments retention factors were calculated according to a classical method already described for FFF experiments on cellular materials [8,9] and peak characteristics (peak width at 50% of the height and asymmetry at 20%) were checked according to the methods described by Bidlingmeyer and Warren [11]. A classical chromatographic pump was used in order to produce a constant flow and a signal-dependent noise. The latter was lightly emphasized to characterize the particles when non-specific spectrophotometric detectors were used. Fractograms obtained in GFFF are therefore shown with a fairly high noise intensity. RBC peaks are characterized by the presence of cells in the eluted carrier phase associated with a noisy response signal.

3. Results and discussion

3.1. Field-flow fractionation of normal red blood cells

Human RBC dimensions are well known, and size descriptions of these cells are available [12]; the average volume of this material is 90 ± 7 μm^3. Because of its discoidal form, other dimensions were observed with an average diameter of 8.1 ± 0.43 μm, the minimum and maximum thicknesses were 1.0 ± 0.30 and 2.4 ± 0.15 μm, respectively, and the surface area was evaluated at 138.0 ± 17 μm^2. Therefore, a sphericity index which describes the morphology was defined; its value is 1 for a pure sphere and 0.77 for a normal RBC [13]. It is also observed that cells with a volume greater than the average value (90 μm^3) are thinner and that those of smaller volume are more spherical. It has also been demonstrated that there is no correlation between the diameter and the smallest thickness [13]. Therefore, the reduction of the RBC to a sphere is a simplification commonly used in FFF. However, the radius of this sphere does not reflect the "size" of the cell. Another well known parameter for describing an RBC is its density. Whereas the volume distribution of RBC is small compared with other blood circulating cells (granulocytes, for example, range from 200 to 350 μm^3), its density distribution is wider and ranges from 1.035 to 1.102 [13]. Any analysis of RBCs with FFF will have to take into account not only the volume, or by simplification the radius of a sphere of equivalent volume, but also the density and the shape of the cell, in particular when a traversal field of low intensity is applied in FFF. Because of its production mode, for a single RBC population, there is a characteristic dispersion in size and in density. By analogy with polymer analysis [14], it is possible to define the RBC as a polymultidisperse population. For example, a given RBC population will have a size, density and shape distribution; rigidity may also have to be described. Based on FFF elution mechanisms, a "pure steric" elution mode [15] would be necessary to elute cells selectively according to an "FFF" equivalent size. Therefore, only FFF techniques able to elute particles in a mode as "steric" as possible [15] can possibly separate RBCs according to an "FFF equivalent size" which can be compared with Coulter volume analysis. FFF methods using a strong transversal field, such as flow [1] and sedimentation FFF techniques, will show under some hydrodynamic and field conditions an "as

steric as possible" elution mode. This principle is described in Fig. 1. However, these methods will neglect an important parameter never taken into account in classical RBC analysis methods, namely the density.

FFF operated at low field therefore opened up a new area, where cells appeared to be eluted according to a "hyperlayer" elution process [16] which involves the density, as shown schematically in Fig. 1. Comparing the RBCs of human origin eluted in the "hyperlayer" mode with latex beads of the same "volume or size" will induce bias, because of size, density, shape and rigidity dispersity of the cellular material. We therefore tried to use normal RBCs of known "average volume" as a standard, using the following assumptions. Human normal RBCs are assumed to show analogous shape and deformity distributions among the different populations under study. We assume also that only differences in size and density will occur. If they only differ in size, in steric and inertial modes, the elution orders will be volume and/or size dependent. If not, discrepancies related to density or other parameters such as shape or deformity will appear. Prior to the experiments, it is necessary to test the reproducibility and define measurement precisions, for this purpose a single reference RBC population was tested in gravitational FFF.

Reproducibility of elution of RBC in gravitational FFF

Samples of normal RBCs, drawn from the same healthy subject under classical haematological control, were analysed at low and high flow-rates. For a retention factor of 0.20, the standard deviation of four measurements was 3%, *i.e.*, a precision of 6% ($\pm 2\sigma$). At high flow-rates, for a retention factor of 0.30, the standard deviation of five measurements was 4%, *i.e.*, a precision of 8% ($\pm 2\sigma$). Retention differences at high and low flow-rates indicated that the elution mode of RBCs in GFFF is not steric, but "inertial" or "hyperlayer". For samples of the same origin, the peak widths at 20% and 50% of the height were measured with a precision of 10%.

Elution mode of RBC in gravitational FFF

The increase in the retention factor of a given particle in a channel of a given geometry with increase in carrier flow-rate was first described by Caldwell *et al.* [17] and was interpreted in terms of lift forces. Kononenko and Simkus [18] described their origin and Williams *et al.* [7] studied their characteristics in SFFF [7]. It appeared that these lift forces depended strongly on the size of the particle (power three of the particle radius) and on the carrier phase density. On the other hand, Martin *et al.* [16] described an elution model that takes account of these lift force to predict qualitatively the elution order of particles of different size and of different density. In the absence of particle wall interactions, which can complicate the elution mechanisms [19–22], or modify them [23], the retention of RBCs, according to these models, will be, among other factors, at least size and density dependent. For example, particles of analogous size but of different density will not co-elute, and the denser particles will be more retained. With SFFF it is possible to modulate the external field, at a given flow-rate, to emphasize a "steric-like" elution mode, *i.e.*, a size-dependent retention. Analogous elution properties are observed in flow FFF [24]. In contrast, with GFFF operating with only one gravitational field, retention modifications according to the density differences of the particles occurred, as described by the "inertial" elution mode established by Martin *et al.* [16] and recently described by Martin and Williams [6]. It is noteworthy that, to date, no systematic work has been able to modify these elution models to take into account particular cell characteristics such as deformity and shape.

Normal RBCs of different average volumes

One trend in the development of FFF is to use this separation technique as a tool to elucidate the size and the size distribution of suspensions. In this regard SFFF appeared, when used at multi-gravitational fields, to be able to analyse in terms of size distributions different submicrometre suspensions. However, the existence of lifting forces [18] and the effect of density were

able to modify the retention [25] of particles. With micrometre-sized particles, SFFF showed a sizing capacity when their densities were known [26], but no demonstration of the sizing power with SFFF for biological particles is yet available. Usually, the density and density distribution of each RBC population are not known, which complicates an "accurate" sizing procedure. It has been shown, with biological particles [27], that a sizing comparison was possible, but no correlation between size or volume estimation classically used in haematology and retention in SFFF was demonstrated. Flow FFF techniques appeared to be more versatile in providing "steric"-like elutions, as demonstrated by Barman and Giddings [28] and Litzèn and Wahlund [24]. Particle wall interactions, lifting forces at low sedimentation field and the density distribution of the cells limited the possible correlation of RBC retention in GFFF and volume estimations given by Coulter techniques. The sizing trial of RBCs by GFFF is also complicated by other factors already suggested [29,30], such as shape and rigidity.

With healthy subjects, systematic elutions of normal RBCs of known average volume and volume distribution were performed in GFFF at high and low flow-rates; the results are shown in Table 1. It was observed that, in fact, no direct correlation between retention and RBC volume was possible in the absence of more complete information on biophysical cell characteristics. These preliminary experiments showed the complexity of sizing RBCs with GFFF. Numerous parameters are involved, as already suggested [12,13,21,28–30], but their relative impacts on retention properties are not known. Therefore, a simple and direct use of FFF for the size estimation of cells must be correlated at least with density (a parameter considered in the "inertial" or hyperlayer elution mode) or other peculiarities that have only just begun to be studied, such as shape [30] and deformity [28]. Taking into account density in particle analysis can be an advantage, as demonstrated by Merino-Dugay *et al.* [10], who used FFF to separate cells of the same size distribution but of different densities. Separation optimization rules in GFFF for RBCs have also been investigated by Urbankova *et al.* [31]. However, it is observed from Table 1 that retention factors are systematically increased when flow-rates are increased, confirming the presence of flow-rate-dependent lifting forces in the elution mode of RBCs [10,27,31].

Table 1
Average volume and retention factor of red blood cells in GFFF

Mean cell volume (μm^3)	Flow-rate (ml/min)	Retention factor ($n = 3$)
57	0.1	0.080
65	0.1	0.060
76	0.1	0.045
99	0.1	0.075
105	0.1	0.077
57	0.5	0.098
70	0.5	0.108
76	0.5	0.092
76	0.5	0.093
89	0.5	0.109
99	0.5	0.102
105	0.5	0.121

Samples were taken from healthy donors. Injection volume, 100 μl; $5 \cdot 10^6$ cells injected at low flow-rates (0.1 ml/min) and $5 \cdot 10^5$ cells at medium flow-rate (0.5 ml/min). Relaxation time, 20 min. Channel dimensions, 915 × 20 × 0.250 mm. Carrier phase, isotonic saline solution (0.9% NaCl). UV detection at 313 nm.

As the elution process of RBCs appeared to be more complicated than a simple "steric" mode, and as it is difficult to define an accurate "hyperlayer" elution model for these cells, we used a methodological procedure to interpret our results. Because of the dispersion of size, density or other characteristics of the RBCs, we used normal RBCs as a standard with the following rules: RBCs of healthy patients are analysed in terms of size and size distributions. Those closer to the average values found in the literature are selected. From these, those with an RDW as close as possible to the "standard values" are selected for the experiments. In the absence of other available parameters, we assumed that their sphericity index and density are also in the "normal" range.

3.2. Field-flow fractionation of pathological cells

The shape, density and rigidity of the cells studied are modified in comparison with normal RBC populations [32]. Retention properties and peak shape analysis will therefore be compared with those for normal cells. Sickle cells appeared to show numerous differences. With respect to normal RBCs, sickle cells are more rigid, with a wider heterogeneity in density and shape [33–35]. The relative intensity of these different parameters is increased with homozygotous (SS) red cells that give irreversible sickle cells during hypoxia, but can be different in each heterozygotous sickle cell case. Red cells from β-thalassaemia subjects are slightly less dense than normal RBCs, as their haemoglobin concentration is decreased, and more spherical than normal RBCs owing to their reduced volume [34]. Therefore, according to the hyperlayer elution mode in GFFF, pathological cells will show different elution properties. Experiments comparing cells of the same average volume, but which differ in density, density distribution, shape, shape distribution and rigidity, will produce modifications in retention. With a pure "steric elution mode" these cells are expected to elute with the same retention factor; in the inertial elution mode and for cells of the same "FFF size", the denser cells will be eluted later.

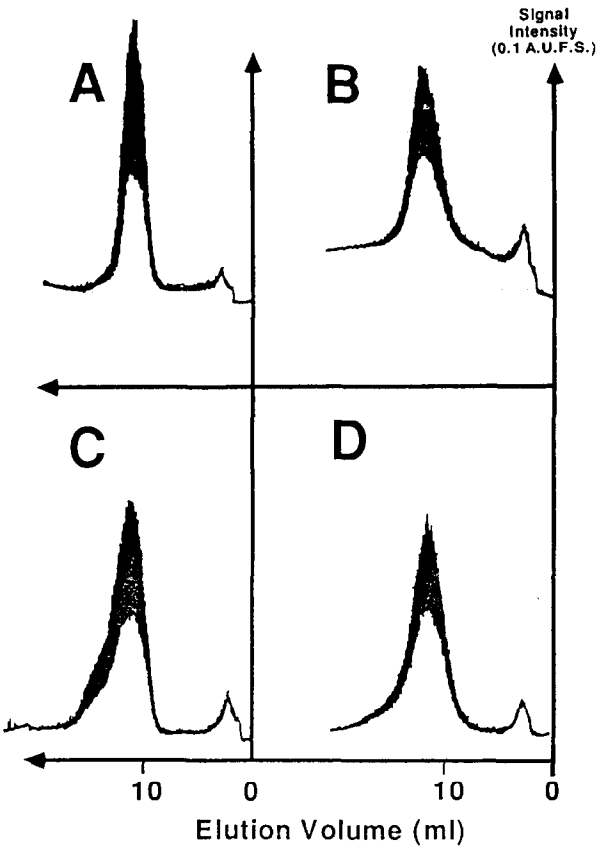

Fig. 2. Normal and heterozygote pathological RBCs. Gravitational field-flow fractionation of RBCs: (A) normal RBCs; (B) thalassaemic cells; (C) and (D) sickle cell anaemia cells. Experimental conditions as in Table 2.

Comparison of heterozygotous pathological cells with normal red blood cells

Samples from two patients presenting heterozygotous sickle cell anaemia were analysed in comparison with a sample from a β-heterozygotous thalassaemic patient and a sample from a normal subject. The normal and sickle cell anaemia RBCs showed an average volume of 90 μm^3 and the β-thalassaemic cell an average volume of 70 μm^3. The fractograms obtained for these four populations are shown in Fig. 2 and the peak characteristics of these four populations are given in Table 2.

Sickle cell trait. It was observed that the sickle cell anemia fractograms (Fig. 2 C and D) show a cell peak less or more retained than a normal RBC population of the same average volume. As already described [33,35], the density distributions of these pathological cells were assumed to be larger than for normal RBCs. The average density of different sickle cell populations could be different from each other. Comparing cells of the same average volume (normal and sickle cells), and considering the precision of the retention factor of 8%, sickle cells of different origins showed strong retention dispersity in FFF, with significant differences as shown in Table 2. Cells of different origins with the same average volume do not elute with the same retention factor for an identical flow-rate. Retention modifications can be generated by the

Table 2
Elution properties of pathological and normal red blood cells

Population	RBC origin	MCV (μm^3)	Retention factor ($n = 3$)	Asymmetry ratio at 20% of the peak height	Ratio of pathological to normal RBC peak variance
A	Normal	90	0.30 ± 0.02	1.1 ± 0.1	1.0 ± 0.1
B	Thalassaemic	70	0.29 ± 0.02	0.8 ± 0.1	1.5 ± 0.1
C	Sickle cell anaemia 1	91	0.37 ± 0.03	0.6 ± 0.1	1.6 ± 0.1
D	Sickle cell anaemia 2	93	0.27 ± 0.02	1.1 ± 0.1	1.4 ± 0.1

Injection and elution conditions: 10^6 cells in 50 μl; 5-min relaxation; flow-rate, 1.0 ml/min; carrier phase, 0.9% (v/v) NaCl, UV detection at 313 nm; channel as described in Table 1.

differences in shape, rigidity or density. If shape and rigidity played the major role, all the different sickle cell populations would be more (or less) retained than the normal RBCs, which is not the case, as observed in Table 2. The other main difference is the density. For cells of average analogous size, *i.e.*, populations A, C and D in Table 2, the sickle cells are not systematically more retained. If we state that retention modifications are density related, sickle cell populations C and D of equivalent volume showed a retention heterogeneity in accordance with the density distribution heterogeneity described in the literature [32–36]. This is confirmed by their asymmetry ratio, which shows a large modification of the peak profile compared with normal RBCs. Moreover, as the sickle cells under study are drawn from heterozygotous patients, we can assume that both their rigidity and their shape distributions are equivalent. Therefore, for equivalent size and equivalent assumed rigidity and shape, their variations in retention will be dictated by density variations. Classical volume or "size" analysis methods with FFF techniques operating at low field are not accurate enough for a diagnosis of the size of cells. The results in Table 2 indicate that for cells of the same average volume, differences are significant when normal RBCs (population A in Table 2), are compared with population C, and differences are not significant when comparing populations A and D. It is therefore possible to state that the average density of population D is of the same order as that of population A. Peak variances of populations A and D or A and C differ significantly, and populations C and D present a wider elution distribution than population A, which is in accordance with the wider density distribution or shape heterogeneity of sickle cells described in the literature [32–36].

Sickle cells from different patients are modified in shape and rigidity when compared with normal RBCs. Moreover, because of their genetic origin, they may differ between themselves (sickle cells C and D are from different patients). To investigate more precisely the origin of these "retention discrepancies", when retention is compared with elution modes established for non-biological particles, another pathology was investigated.

Thalassaemic cells. With β-thalassaemic cells, the lower average volume is associated with a decrease in density and a higher sphericity index [34]. In Table 2, no significant differences were observed in retention between population A (normal) and population B (thalassaemic) for a significant modification of the volume (90–70 μm^3). According to the elution modes expected for micrometre particles, *e.g.*, the steric elution mode, the thalassaemic cells, with a lower volume, would be eluted with a lower retention factor. However, thalassaemic cells are less dense and their elution rate according to the hyperlayer elution process may increase. The retention difference due to the volume is balanced by the density variation. The more homogenous shape distribution of mature thalassaemic cells compared with normal cells or sickle cells is balanced by an increased proportion of

reticulocytes [34], which explains the increased peak variance. Significant differences observed for the asymmetry factor may also be of the same origin.

If one uses GFFF to measure only the size of the particles, this technique is not accurate. Nevertheless, GFFF provides additional and original information on the cell characteristics. Not only is the retention affected, there are possibilities of correlating peak variance and asymmetry with size and/or density or shape. In all the heterozygote cells obtained in sickle cell anaemia and thalassaemia, the elution profile of pathological cell dilution showed a systematic peak volume increase measured at 50% of the peak height. This in accordance with the increased heterogeneity in density, shape or morphology (reticulocytes) described in the literature [32–36].

Sickle cell anaemia: application to the monitoring of a therapeutic process

When a child is diagnosed to have a sickle cell anaemia, the blood of the patient may be partially replaced by transfusion. A 4-year-old child included in the present study with sickle cell anaemia was transfused according to a particular protocol: 390 ml of his blood was drawn and replaced with 290 ml of concentrated normal RBCs and by 100 ml of plasma substitute. This blood replacement, also called "exchange", modified the RBC composition of the patient's blood, reducing the 100% S haemoglobin to 37%. In such a procedure, two RBC populations were therefore present in the blood stream.

This "exchange" procedure was monitored by GFFF. Three fractograms were studied and are shown in Fig. 3. A normal RBC population was used as standard (Fig. 3A), and the elution of the pathological blood before and after transfusion was performed (Fig. 3C and B). A shown in Tables 2 and 3, normal cells of the same origin eluted under the same conditions showed analogous peak retention and peak profiles.

The retention factor of the sickle cell anaemia cells is higher than for the reference RBCs, as shown in Table 3, which is in accordance with the density increase of sickle cells. When the

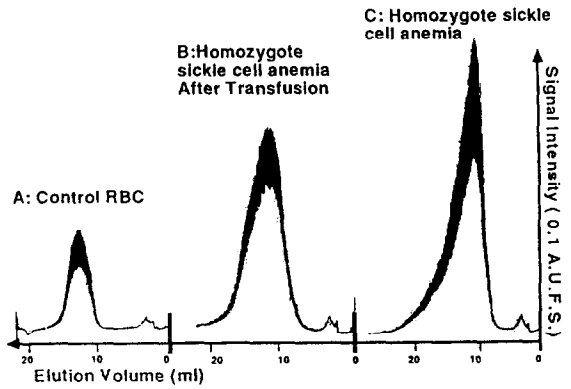

Fig. 3. Normal and sickle cell anaemia cells before and after transfusion. Gravitational field-flow fractionation of RBCs: (A) control RBCs; (B) homozygote sickle cell anaemia cells after transfusion; (C) sickle cell anaemia cells before transfusion. Experimental conditions as in Table 3.

asymmetry ratio and peak variance are compared with those for normal RBCs, a systematic and significant increase is observed, which indicates an increase of density and shape heterogeneity. Comparing populations C in Table 3 with populations C and D in Table 2, the asymmetry ratio is increased, which suggests that the sickle cells for homozygotes are more heterogenous than those for heterozygotes, as described in the literature [34]. A lower peak variance for population C in Table 3 compared with populations C and D in Table 2 can be interpreted in terms of heterogeneity distribution (heterogeneity distribution was not described as Gaussian).

Under the elution conditions used here (channel thickness 250 μm), the "exchange" procedure did not show the appearance of a double RBC population, in contrast to other transfusion processes already described [9], in channels of smaller thickness. The peak variance is strongly increased and the peak asymmetry is modified, which confirms that the characteristics of population B in Table 3 are intermediate between those of populations A and C in Table 3. The retention factor of population B in Table 3 is analogous to that of the normal RBC population. The eluted population is a mixture of sickle cells and of normal RBCs. In this mixture, the transfused cells are in the majority, and therefore the

Table 3
Elution properties of sickle cell anaemia cells before and after transfusion

Population	RBC origin	MCV (μm^3)	Retention factor ($n = 3$)	Asymmetry ratio at 20% of the peak height	Ratio of pathological to normal RBC peak variance
A	Normal	91	0.28 ± 0.03	0.92 ± 0.09	1.0 ± 0.10
	Sickle cell anaemia				
B	After transfusion	91	0.29 ± 0.03	0.60 ± 0.06	1.7 ± 0.10
C	Before transfusion	93	0.33 ± 0.03	0.40 ± 0.03	1.3 ± 0.1

Injection and elution conditions: 10^6 cells in 50 μl; 5-min relaxation; flow-rate 0.95 ml/min; carrier phase, 0.9% (v/v) NaCl; UV detection at 313 nm; channel as described in Table 1.

retention observed is analogous to that observed for normal RBCs, and the remaining pathological cells were responsible for the increase in variance if population C in Table 3 is compared with normal RBC (population A).

3.3. Density assessment in FFF studies

In this work, literature values for shape and density or density distribution were used to interpret the results; density data widely studied for RBCs seem to us methodologically more accurate than the results of a specific experimental density gradient procedure. Two points should be made. First, in terms of the separation, FFF will compete with the gradient centrifugation technique, the major advantage being the use of simpler carrier phases. Second, the need for density assessment in FFF is due to the role played by density in the elution mode of micrometre-sized particles in FFF. The roles of density and, the density and size balance in the hyperlayer elution mode have not yet been completely assessed, with an inertial or lubrication origin [6,7,16,37]. This is why control procedures using density gradient are needed. On the other hand, as shown in a previous report [10], relaxation injection procedures can give information on density, but the time-consuming procedure (series of injections at different stop-flow times) and the cell storage conditions can lower the accuracy of such measurements, in particular when human pathological cells are involved. This problem is also encountered with the gradient centrifugation technique.

The use of standards is possible; latex beads monodisperse in size are commercially available, as are beads monodisperse in density. No series of particles monodisperse in size and density are available, limiting comparisons of the elution of RBCs with standards. New complications will arise when shape or rigidity are taken into account.

4. Conclusions

The low field used in gravitational FFF and the hyperlayer elution modes involving only size and density partially explain the data presented in this paper. It appeared that the shape and deformity would also be responsible for the retention properties of RBCs [28–30]. GFFF seems to have limited applications in terms of size analysis compared with flow FFF techniques [28]. However, this limitation creates advantages: with gravitational FFF, density variations can be shown and, combined with "relaxation process" studies [10], FFF may produce additional information on cell characteristics. Previous reports have demonstrated that normal RBCs can be separated in some instances according to the size, if size differences are significant [9,10,27]. If the size of different sub-populations is the same, cells can be separated according to their density [10]. In contrast to simple size measurements, gravitational FFF emerged as a versatile tool for studying cells. For example, the retention dispersion observed for sickle cells of the same volume and of higher density

and shape heterogeneity compared with normal RBCs is demonstrative. Cell elution in GFFF appeared more complex than a simple size and density mechanism. However, the effects of shape and rigidity would have to be studied at constant volume and density in the steric or inertial elution mode [28,30]. Even with its relatively low efficiency in terms of size measurements, in gravitational FFF numerous parameters can be analysed, and optimization procedures are needed for a better understanding of the relative effects of size, density, shape and rigidity of the cell elution characteristics. The "exchange" transfusion of sickle cell anaemia illustrates the monitoring power of gravitational FFF in clinical biology studies. However, the results reported in this paper were obtained using a fairly wide channel (250 μm) to allow comparison with other FFF techniques using the same thickness. According to steric and inertial models, thinner channels will emphasize density or size differences [37,38]; correlations between size and retention with FFF operated at high field have been recently published [28], and will therefore allow more precise analyses of shape or rigidity.

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