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Monitoring of an experimental red blood cell pathology with gravitational field-flow fractionation

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ABSTRACT

Gravitational field-flow fractionation is a simple method suitable for the separation of micrometre-size particles, for example the red blood cells (RBC). The variation in the composition of the RBC population in a rabbit during an experimental reversible anaemia induced by phenylhydrazine was studied. Blood samples taken at different stages of the aenemia showed differences in the retention and shape of the elution profiles. Microscopic observations of original RBC samples and fractions collected at the outlet of the fractionation channel make a description of the RBC population possible. The three species observed were the normal RBC, the newly produced reticulocytes, and the blood cells containing Heinz bodies (intracellular haemoglobin precipitates). A decrease of the normal RBC from 96 to 1% was observed over five days. The production of reticulocytes in bone marrow is stimulated by the anaemia and increases in percentage after the second day of the anaemia (from 1 to 16%). RBC with Heinz bodies, which appear on the third day, were also studied. Granulometric studies were performed on the RBC sampled from the rabbit each day as well as on some fractions eluted by field-flow fractionation. Reinjection procedures of some cell subpopulations of known size distribution were also performed. The relaxation process of these cells was then studied to approximate their density properties. It was observed that RBC of different density but of the same average size were selectively eluted, as were cells of equivalent density but of different size. Injection of the cells at different stop-flow times enabled the study of the relaxation process on the elution profiles. The results, compared with systematic microscopic observation and size analysis, permit the description of modifications in the RBC composition as well as the purification of subpopulations at each stage of the anaemia. The correlation observed between the fractionation profiles and the progress or the regression of the anaemia opens a new field in the analytical monitoring of this type of pathology.

INTRODUCTION

Studies of the elution properties of red blood cells (RBC) in field-flow fractionation (FFF) have been performed for numerous years [1,2]. With some restrictions, fundamental experience gained with latex particles can be applied to this cellular material as well as other biological systems for studies of FFF performance in biology. It has been demonstrated that non-biological particles can be selectively eluted if they differ in size, density and shape [3–5]. In the case of human RBC, selective elution of cell subpopulations has also been demonstrated [6], and it was shown that size and density and possibly cell

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shape, were involved. It is then possible to monitor modifications of cell populations by FFF techniques and to correlate the characteristics of the cells with their elution profiles.

A well established, reversible model of red cell change is provided by haemolytic anaemia induced in rabbits by phenylhydrazine [7]. This process, by simultaneous steps of haemolysis of the RBC in the peripheral circulation and regeneration in the haematopoïetic bone marrow, will modify the RBC proportions of four characteristic subpopulations, which are easy to characterize by microscopic observation. The four populations are: normal RBC, reticulocytes and two types of cell that include in their cytoplasma Heinz bodies (haemoglobin precipitates) at levels below or above ten inclusions per cell. Investigation of this anaemia with the FFF technique can open new opportunities in cell purification, and will allow an improved type of description of the modifications of RBC populations.

The principles of FFF elution mechanisms have been extensively described [3–5,8,9]. To date, three elution models are accepted (normal, steric and inertial, also called hyperlayer). It has also been demonstrated that the retention of RBC in gravitational FFF appears to be a process that includes both the steric and the inertial models [2,6,10]. In such a process, it has been demonstrated that the separation depends on both particle density and particle size. Therefore it is possible to separate particles of the same density and of different size as well as particles with the same average size but of different density.

We have investigated the gravitational FFF behaviour of living rabbit RBC during an eight-day period. The anaemia was induced the first four days by phenylhydrazine. In the blood circulation, phenylhydrazine induces an oxidative denaturation of haemoglobin. This denaturation becomes visible by the presence of Heinz bodies in the RBC. Such cells do not transport any functional haemoglobin in the circulation, and they are known to be easily trapped and destroyed in the spleen. The strong decrease of functional haemoglobin levels stimulates the production of new RBC in the bone marrow. This stimulation is evidenced by an increase in the number of very young RBC, called the reticulocytes. Under phenylhydrazine treatment the turnover of RBC is accelerated by a simultaneous destruction and production of cells, which modifies the RBC composition during and after the treatment, leading to a population with an average age lower than before the treatment. FFF elution experiments, microscopic observations and Coulter counter size measurements were used to monitor the RBC cell population during the haemolytic anaemia.

EXPERIMENTAL

Field-flow fractionation

The separation channel was built similarly to the ones already described by Giddings and Myers [11]. Two mirror-quality glass plates, which enclosed a Mylar band, were compressed by two screwed plexiglass plates. The Mylar band was cut to produce a rectangular channel. To meet the special conditions required for living biological material, the glass plates (channel walls) were coated with biocompatible silicone (Silbione, Rhône Poulenc, Paris, France). The void volume of the channel, including the tubing connections, is 3.16 ml, and the dimensions of the channel were 85.5 × 2 × 0.0175 cm. The carrier mobile phase and sample dilution solvent was an isotonic phosphate buffer (pH 7.4).

Most of the experiments described in this report were performed with the same injection protocol. In the abscence of flow, 100 or 50 μl of the suspension containing from *ca.* 200 000 to *ca.* 400 000 RBC were injected through a septum device in the channel. The particles settled during a given time (usually 4 min) in the thickness of the channel, then the flow was established using a Waters 6000A pump. Detection was performed with a photometric detector set at 313 nm (Shimadzu SP6A). The fractogram parameters were determined by classical methods [1,2,12]. The peak profile accuracy and the precision in work with biological materials were established with human RBC of known characteristics. The results

TABLE I

PEAK CHARACTERISTICS: PRECISION MEASUREMENTS

Channel thickness, 0.175 mm; injection with relaxation; 200 000 particles in 50 μl; precision, ± 2σ.

	Reproducibility over 24 h[a]				Day-to-day reproducibility[b]
	Retention factor	Peak width	Asymmetry factor	Peak height	Retention factor
Latex beads	0.15 ± 0.002	5.2 ± 0.10	1.6 ± 0.08	15 ± 0.8	0.19 ± 0.007
Red blood cells	0.28 ± 0.03	2.4 ± 0.05	0.93 ± 0.020	–	0.28 ± 0.012

[a] Latex beads flow-rate, 0.69 cm/s; RBC flow-rate, 0.43 cm/s; $n = 4$.

[b] Latex beads flow-rate, 0.74 cm/s; RBC flow-rate, 0.43 cm/s; $n = 14$ (two measurements per day for seven days).

of this validation process are shown in Table I, in comparison with latex beads of equivalent particle volume.

Red blood cells

Haemolytic anaemia was induced in a New Zealand white rabbit (2.24 kg) by daily subcutaneous injections of phenylhydrazine (8.75 mg/kg) for four days. The sample of day 1 was taken just before the first drug injection, and other samples were taken on days 3, 4, 5 and 8. Each day, the blood was drawn into tubes containing EDTA as an anticoagulant. The cell volume distribution was analysed, using the resistivity detector of a Model ZM multi-channel Coulter counter set for 64-channel analysis (Coultronics, Margency, France). All the size histograms included in this report show the number of cells expressed as a percentage of the total analysed population. The average concentration of cells in the blood stream was evaluated each day by the measure of the haematocrit ratio (volume of the cell population to the sample volume). This procedure allowed the calculation of appropriate dilutions for Coulter counting. With the use of direct optical microscopy, a cell count was made on blood films stained with May–Grunewald–Giemsa [13] or Crystal Violet [13,14] (cells with Heinz bodies) or Brilliant Cresyl Blue [13,14] (reticulocytes).

RESULTS AND DISCUSSION

Reversible anaemia induces modifications in the composition of the RBC population, which can be described by classical haematological methods, such as morphological description under microscopical observation and size analysis by Coulter counting. This report shows that complementary information is obtained when the anaemia is monitored with these classical methods and with FFF. The separation power of FFF means that the purification can be optimized by fraction collection and reinjection.

Modifications of retention and shape characteristics of elution profiles

During haemolysis, by the action of phenylhydrazine and concurrent regeneration, fractograms of RBC were obtained as shown in Fig. 1. The first two peaks are observed, in all the fractograms: they can be considered as "system peaks". They generally correspond to species not affected by the external field and their origin has been explained elsewhere [2,15]. The third peak, which can appear bimodal, corresponds to the RBC. The composition variation of the RBC for eight days was described for the four most important subpopulations, which are known to be strongly modified in number by the anaemia: the normal RBC, the reticulocytes and the two populations showing either more or less than ten Heinz bodies per cell. The composition modifications of these subpopulations during the regenerative anaemia are described in Fig. 2.

On day 1, the RBC were eluted as a single peak (Fig. 1), and the global analysis of the blood sam-

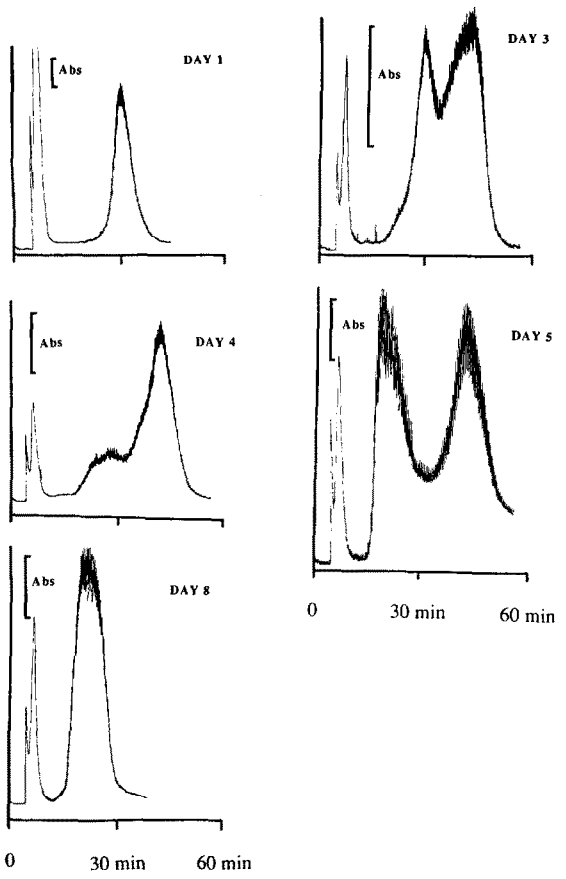

Fig. 1. Fractograms of rabbit RBC before, during and after phenylhydrazine-induced anaemia. A 100-μl sample of RBC suspension of 4000 cells per μl injected with a 4-min stop-flow time. Carrier phase, isotonic phosphate buffer (pH 7.4); flow-rate, 0.21 cm/s; detection wavelength, 313 nm. Abs shown on fractograms is $6.5 \cdot 10^{-3}$ absorbance units.

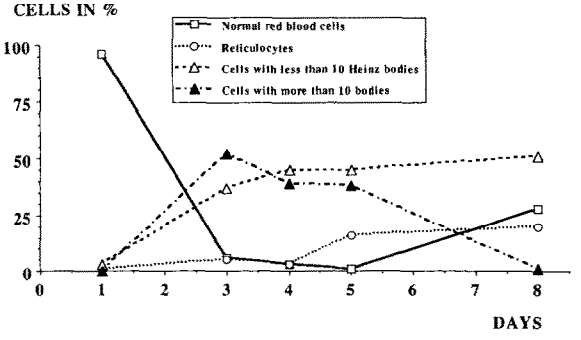

Fig. 2. Composition modifications of RBC population during the experimental haemolytic anaemia. RBC samples were analysed before FFF elution. Samples were stained with May Grunwald Giemsa, Crystal Violet and Brilliant Cresyl Blue. Subpopulation characterization was performed by statistically significant direct counting with optical microscopy magnification ($\times$ 100).

ple showed the presence of 96% of normal RBC (Fig. 2), the remaining 4% being reticulocytes and old RBC with a single Heinz body. This symmetrical profile is characteristic of a normal RBC population and matches those published previously for both human [1,2] and animal [2,16]

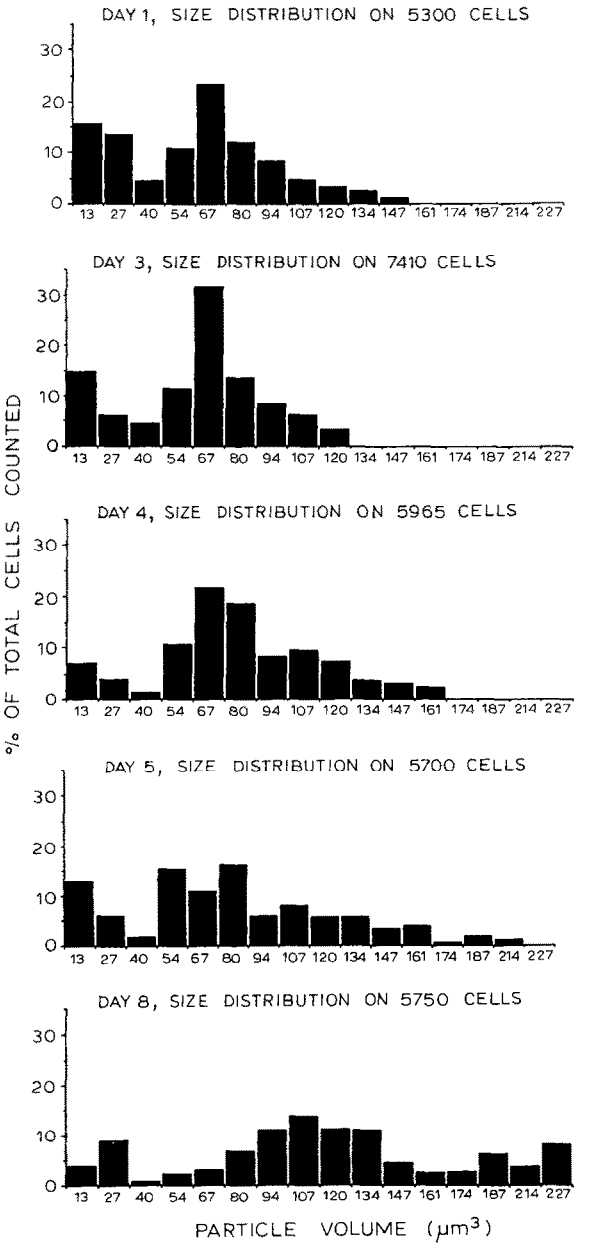

Fig. 3. Size distribution of rabbit RBC sampled during haemolysis. Coulter counter Model ZM set for a 64-channel analysis. Calibration of the system with 5.6-μm-diameter latex particles.

cells. In these elution conditions, the morphological observation of peak fractions (beginning, middle, tail) by microscopical observation did not show any enrichment in either subpopulation [17]. Under the elution conditions described in the legend of Fig. 1, a retention factor of 0.21 was obtained for the RBC peak eluted on day 1. The retention factor (R) is defined as the void volume of the separation system divided by the retention volume.

On the first three days, because the phenylhydrazine-induced anaemia has been acting for 48 h, the haematocrit measurement showed a decrease of 5% per day of the RBC population. On day 3, the normal RBC population had dropped from 96% on the first day to only 6%, and 89% of the cells contained denatured haemoglobin. In 37% of the cells between one and ten Heinz bodies had been formed, and 52% contained more than ten. The elution of RBC sampled that day (day 3) produced a fractogram with two badly resolved peaks, one with a retention factor of 0.24 and the other with $R = 0.17$ (Fig. 1). A morphological analysis of the cell composition of the less retained peak ($R = 0.24$) showed essentially cells with one Heinz body (60% of the cells counted in the fraction) and a non-negligible proportion of reticulocytes. The second peak ($R = 0.17$) was noticeably broader and contained all the other species. Two thirds of this fraction were made up of species with more than one Heinz body.

On day 4, the total blood composition was similar to that of day 3, but the majority of the Heinz cells showed more than eight granulations. It was also observed, on the fractogram of RBC sampled that day, that the height and shape of the first RBC peak had been modified.

On day 5, 24 h after the phenylhydrazine injections had been stopped, there was a significant increase in the reticulocyte concentration. Fraction analysis of the bimodal peak eluted that day indicated that these reticulocytes were found primarily in the first peak. In the second peak, cells with Heinz bodies were observed and represent *ca.* 80% of the cell population found in that peak.

On day 8, the composition of the blood sample before FFF elution contained 30% of normal RBC and 20% of reticulocytes, most of the other cells containing less than ten Heinz bodies. The fractogram profile was analogous to that of day 1. It was observed that the retention factor ($R = 0.31$) of this rabbit RBC population was higher than that measured on day 1.

Because modifications in the RBC composition caused the appearance of new peaks, retention modifications of RBC peaks, and modifications in the peak shape, a complementary analysis of the cells before and after elution was performed. In haematology and in particle analysis, size estimations are widely used, but density evaluations may also be needed [18].

Selective size elution of RBC subpopulations

Size analysis of the RBC populations was performed before elutions and in some cases after collection of fractions (day 4, day 5). Fig. 3 shows the histograms of the size distributions of the RBC sampled on days 1, 3, 4, 5 and 8; the volume distribution is given in percentage of the cell number. One can observe day-to-day variations in the granulometric distribution of the RBC. It can be observed that the RBC of day 8 have an average size (120 μm^3) larger than those of day 1 (71 μm^3). Fig. 3 shows that on day 8 the volume distribution is shifted to larger volumes. These results have to be combined with the fact that the retention factor is higher on day 8 than on day 1 ($R = 0.31$ and 0.21, respectively). If factors such as the density and shape or rigidity of the cells are ignored, we can conclude that in this case the cells of larger average volume (day 8) are eluted faster than the smaller ones (day 1).

On day 4, a bimodal peak profile is observed and two fractions are collected, the first corresponding to the cells eluted at a retention factor of 0.24 (start 20 min, end 30 min) and the second to the cells eluted from 30 to 60 min (retention factor 0.17). The size histogram of the original sample injected is shown in Fig. 3 (day 4) and gives an average volume for the cells of 71 μm^3. Note that more than 50% of the cells were in the range 60–90 μm^3. Fig. 4A shows the size histo-

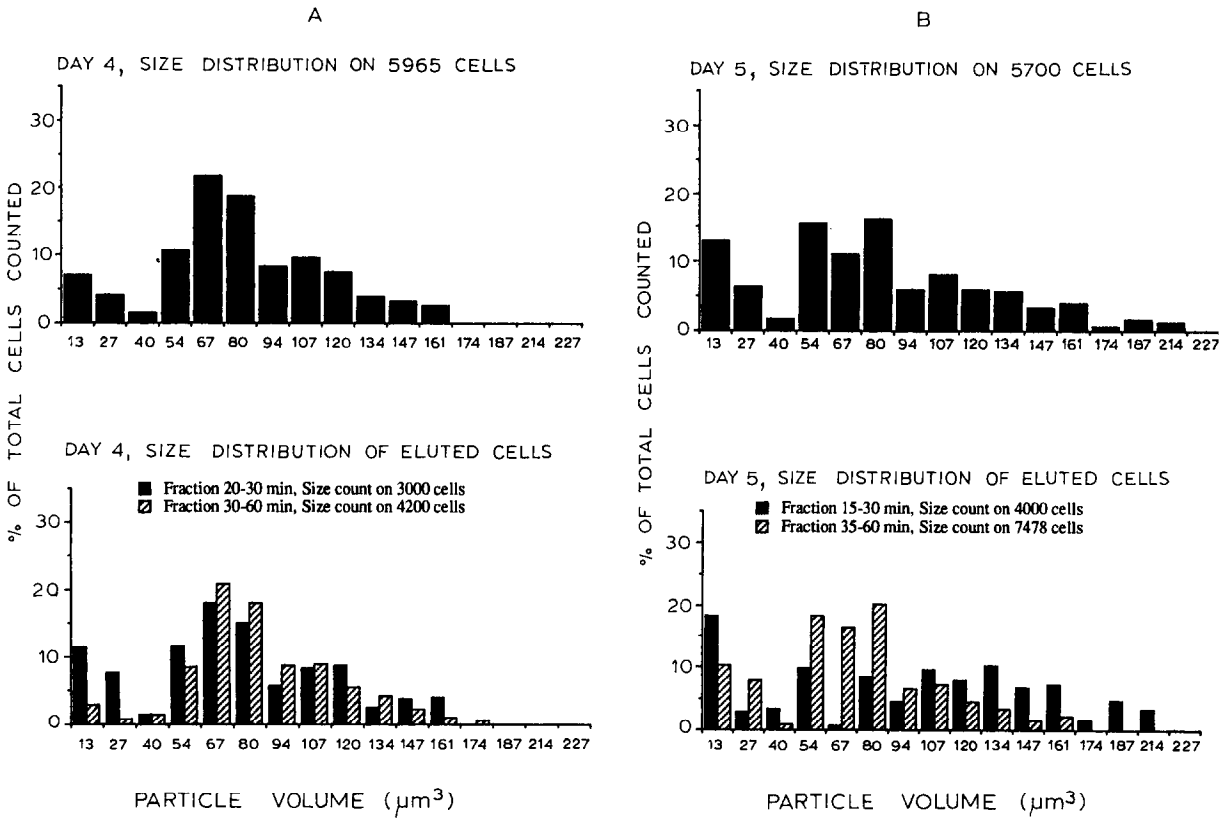

Fig. 4. Size distribution of RBC. (A) Rabbit RBC sampled before (upper) and after (lower) elution on day 4. (B) Rabbit RBC sampled on day 5, before (upper) and after (lower) elution. Coulter counter Model ZM set for a 64-channel analysis. Calibration of the system with 5.6-μm-diameter latex particles. The upper graphs show the sample before injection, and the lower graphs show the size distributions of the two purified fractions collected after FFF elution.

gram of the two FFF-eluted RBC subpopulations, compared with the original one. On day 4, size evaluation of both populations separated by FFF give an average volume of 71 μm^3 for the population collected first (retention factor 0.24) and an average volume of 74 μm^3 for the fraction eluting later: in both cases, more than 50% of the cells were in the range 60–90 μm^3. This average volume difference is hard to detect by visual inspection of the figure and it may not be significant.

Because of the presence again of a bimodal peak on day 5 two fractions were collected, the first including the cells eluted between 15 min and 30 min and the second between 35 min and 60 min. Size analysis of both fractions was performed and the size distribution histograms are shown in Fig. 4B. It was observed that the two fractions show differences in their size distributions. The cells eluted first (15–30 min) have an average volume of 120 μm^3 in a broad distribution, with more than 50% of the cells in the range 100–160 μm^3. The cells eluted after 35 min were smaller, with an average volume of 67 μm^3. These results demonstrate that the selectivity in the retention of RBC can be due to differences in size, as also indicated by the comparison of retentions obtained on day 1 and on day 8. The elution process appeared to follow a size-dependent elution order, often described as "steric" [1], in which the bigger particles were eluted before the smaller ones.

The size measurements performed on day 4, however, do not show similar results, which in-

dicates that the size cannot be the only parameter involved. As described previously [2,6,19], density can be taken into account, and was to be evaluated for a more complete comprehension of the elution mechanism of the RBC. Classical methods of density determination are related to density centrifugations [20]. Since these experiments take a relatively long time, subjecting the cells to stress and possibly jeopardizing recovery, we have tried to compare the density of the cells by FFF using the concept of "relaxation time" [21].

Relaxation and particle characterization

One critical step in achieving a separation by FFF is the relaxation process [21,22]. To permit particles to reach their equilibrium position in the separation channel, it is recommended that the samples are injected into the mobile phase at stopped flow and that the flow is resumed after a period of a few minutes. The time needed to allow complete relaxation depends on the channel thickness, the particle size and density, and the carrier phase density. It is possible to determine the relaxation time of a given particle by a series of experiments performed at different stop-flow times.

As the relaxation time is used to describe the transversal equilibrium state reached by the cells in the channel, a stop-flow period longer than the relaxation time will not produce any variations in the elution characteristics. On the other hand, as long as the elution profile changes with stop-flow times, one can consider that this equilibrium state has not yet been reached. The experimental relaxation time can be defined as the shortest stop-flow time where no more modifications of the peak characteristics occur [23]. Three parameters have been studied on human and rabbit RBC fractograms: the retention factor, the peak width and the asymmetry factor. Results obtained with human and rabbit RBC are shown in Fig. 5 and 6.

To determine the equilibrium position, a weighted first-order polynomial least-squares fitting procedure has been used ($R = f(1/t)$). Because of the large number of experiments (eighteen elutions in the case of human RBC) and to emphasize the importance of high stop-flow time experiments, the weighting procedure was time-dependent. The retention factor, the asymmetry ratio and the peak width can be extrapolated to obtain the limiting retention factor, the limiting asymmetry ratio and the limiting peak width to an infinite stop-flow time. These values are plotted in Figs. 5 and 6. Even if altered by possible systematic errors (related to equilibrium and sedimentation) these values will be considered in this

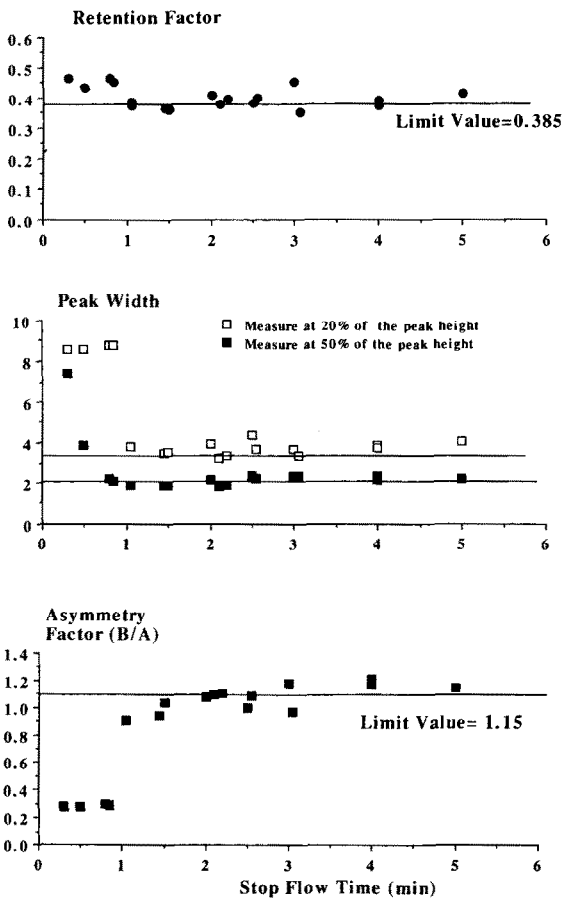

Fig. 5. Fractogram elution characteristics of normal human RBC obtained at different stop-flow times. The retention factor is calculated as the ratio of the elution time of non-retained species (proteins) to the elution time of the summit of the peak. Asymmetry factor ratio (B/A) is measured at 20% of peak height. Peak width is given in minutes and measured at 50% of the peak height. Injection conditions: 50 μl of a suspension of human RBC containing 4500 cells per μl diluted in an isotonic buffer. Flow-rate 0.48 cm/s. Average volume of RBC used, 90 μm^3.

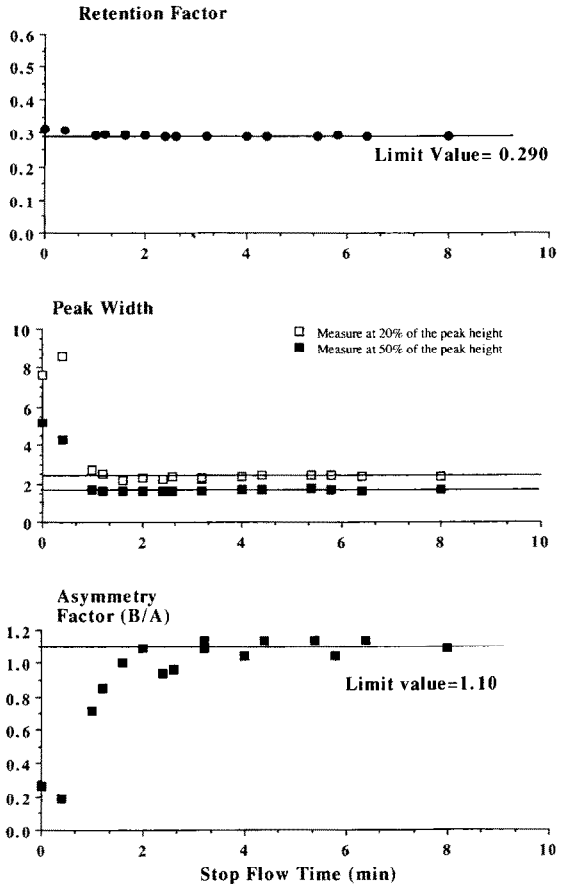

Fig. 6. Fractogram elution characteristics of normal rabbit RBC obtained at different stop-flow times. Measurement protocol identical to Fig. 5. Injection of 200 000 cells in 50 μl. Flow-rate, 0.48 cm/s. Average volume of RBC, 71 μm^3.

report as a probe for the relaxation time [21]. It should be noted that all the experiments described in this report were performed under similar conditions.

To determine the minimum stop-flow time, linear interpolations between successive data obtained at increasing stop-flow times are performed, and the first intersection with the lines corresponding to values extrapolated at infinite stop-flow times will be considered as the relaxation time. Depending on the peak characteristic used, at least three values are obtained. In the case of normal human RBC used in these experiments, the "relaxed" values are: R = 0.385; peak width at 50% = 2.05; peak width at 20% = 3.38; asymmetry ratio = 1.15. This leads to four relaxation time values of 1.05, 2.1, 2.2 and 2.55 min, respectively, and the average relaxation value was 1.98 ± 0.6 min.

It is then possible to compare these experimental values with the ones calculated using the analytical expression of the sedimentation time, described in FFF by Giddings *et al.* [21]. For human RBC, the values given in the literature [14,15,18] are a density (ρ_p) of $1.086 < \rho_p < 1.117$, and an average volume of 90 μm^3 (2.8 μm for the radius of spherical particle of equivalent volume). Based on these values, the sedimentation time needed for a particle to settle in the channel

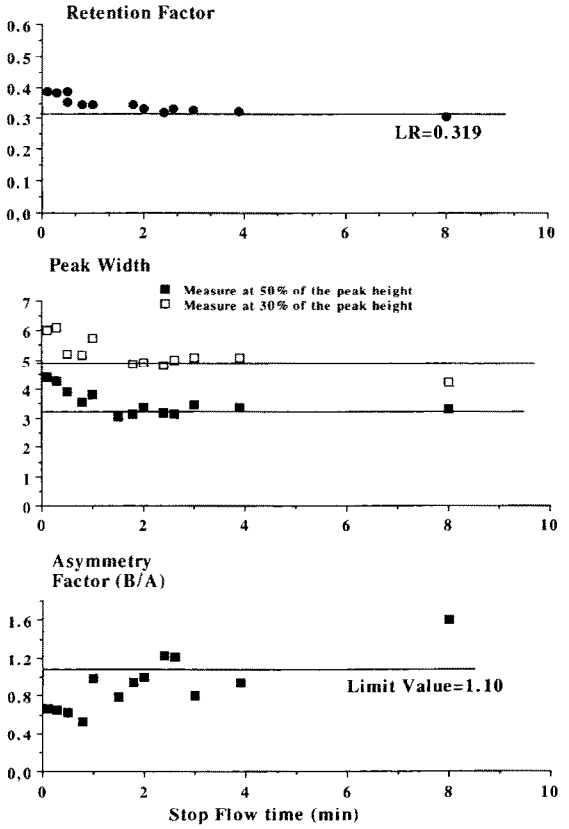

Fig. 7. Fractogram elution characteristics of rabbit RBC fraction 1 on day 4, obtained at different stop-flow times. Fraction 1 was eluted between 20 and 30 min on day 4. Four cumulative elutions were performed to collect cells of fraction 1, and cells were collected manually under time and signal control. The retention values shown in the figure were measured for each peak after reinjection. An average of 200 000 cells were injected with different stop-flow times in a 60-μl carrier phase volume. The ordinate is the percentage of the total number of particles counted.

would be in the range 1.50–2.13 min. Even if not precise, the average relaxation value determination is in accordance with the literature data.

The same experiments were applied to normal rabbit RBC. Coulter counting indicated an average volume of 71 μm^3, and the estimated average relaxation time was 2.2 ± 0.4 min. To determine the characteristics of the cells eluted on day 4, which showed analogous size distributions, reinjection and elution procedures were applied at various stop-flow times. The fraction eluted first showed an estimated average relaxation time of 2.2 ± 0.3 min (Fig. 7), whereas for the more retained cells a 20% shorter relaxation time of only 1.78 ± 0.5 min was found (Fig. 8). Because the particle volumes of the two fractions (71 and 74 μm^3, respectively) are practically identical in terms of size distribution, and because the average volumes do not present any significant differences, the different sedimentation behaviour can be interpreted as due to differences in the densities of the cells. According to Giddings *et al.* [21], the sedimentation time decreases with increasing density of the particles, so the faster sedimentation of the RBC of the more retained fraction suggested that these particles had a higher density than the cells in the first fraction.

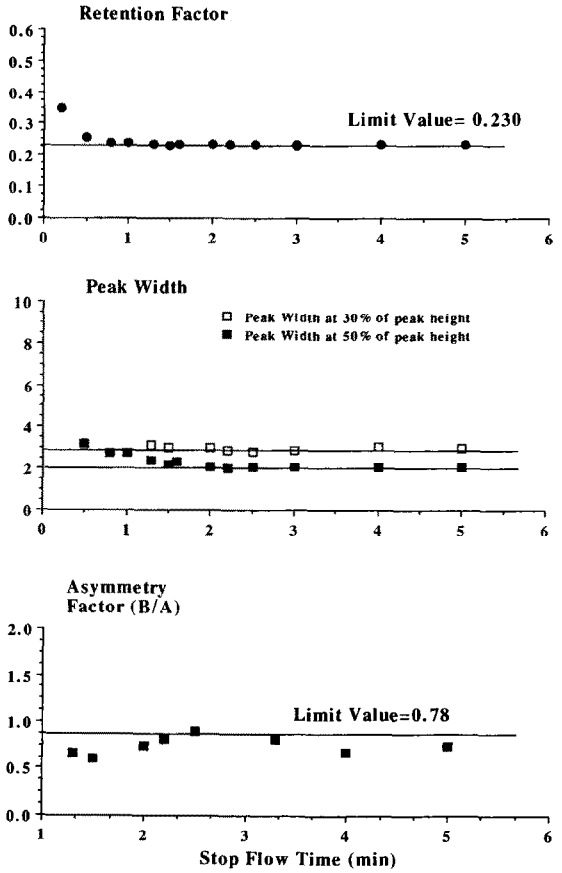

Fig. 8. Fractogram elution characteristics of rabbit RBC fraction 2 on day 4, obtained at different stop-flow times. Fraction 2 was eluted between 30 and 60 min on day 4. Four cumulative elutions were performed to collect cells of fraction 2, and cells were collected manually under time and signal control. The retention values shown in the figure were measured for each peak after reinjection. An average of 200 000 cells were injected with different stop-flow times in a 60-μl carrier phase volume.

FFF study of particle properties: flow-rate effects on the retention

Previous publications describe a variation of the retention with the flow-rate and attribute this effect to a so-called lifting force (Giddings [24]) or hydrodynamic or inertial forces (Martin and Reynaud [4] and Kononenko and Shimkus [5]). In order to obtain a more precise evaluation of the biophysical properties of the cells, and to investigate these lifting forces, systematic studies of retention *versus* flow-rate were performed on some rabbit RBC populations.

Fig. 9 shows a parallel variation of the retention of samples from days 1, 4 and 8. The bigger particles (day 8) are systematically eluted with a retention factor higher than that of the smaller ones (day 1). The results for the first fraction (20–30 min) of day 4 are intermediate between those for the other two curves (days 1 and 8). Comparing this population with the one of day 1, it is observed that they differ significantly, although the average size of both populations was of the same order (71 μm^3).

One parameter to take into account is the density of the particles, which, together with the size, determines the strength of the lifting forces [4,5,15,24]. The measurement of the relaxation time indicated that, at an equivalent size, the least dense particles are eluted first (subpopulations of day 4). It can be stated that the cells of day 1 are denser than those in the first eluted fraction of

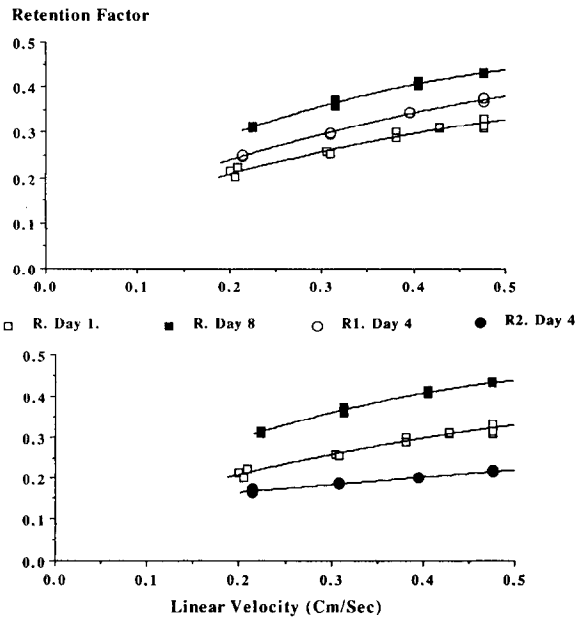

Fig. 9. Effect of flow-rate on the retention of rabbit RBC. Reinjection as in Fig. 4 of the two populations purified by FFF elutions on day 4: R1, 0.24; R2, 0.17.

day 4. The retention variation of the second fraction of day 4 (described in the legend of Fig. 9 by R2, day 4) differed in the slope of the retention *versus* flow curve. The lifting forces acting on these cells can be considered to be different from those acting on the first fraction of day 4 or on the populations sampled on days 1 and 8.

CONCLUSION

Fractogram profiles of RBC depend strongly on the cell population characteristics. The gravitational FFF technique is able to elute selectively cells of different sizes (day 1 and day 8, subpopulations of day 5). At the same time, it can separate species according to their densities, as observed for the subpopulations of day 4: for an equivalent size, the denser the cell, the lower the retention factor. This size and density selectivity of the simple gravitational FFF technique is a powerful tool for the separation of micrometre-size particles, such as RBC. In particular, it is possible to correlate direct microscopic observations with cell retention [17]. The use of the stop-flow time injection protocol to establish the characteristics of the eluted cells, even with some lack of precision, is of interest because the density study performed in that case is made in a single gravitational field and in an isotonic buffer, contrary to the more classical density-gradient centrifugations. As shown in this report, with relaxation, young rabbit RBC eluted with a retention factor higher than the older RBC. In the case of injections of human RBC in the flow, older cells eluted before younger ones [6]. These results suggest that retention or elution orders may be modified according to the complexity of the separation process of biological material of different origins in gravitational FFF. Improvements in the methodology, such as the use of centrifugal multigravitational FFF systems, can be expected to reduce measurement times and increase precision and accuracy.

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REFERENCES

1 K. D. Caldwell, Z. Q. Cheng, P. Hradecky and J. C. Giddings, *Cell. Biophys.*, 6 (1984) 213.
2 Ph. J. P. Cardot, J. Gerota and M. Martin, *J. Chromatogr.*, 568 (1991) 93.
3 K. D. Caldwell, T. T. Nguyen, M. N. Myers and J. C. Giddings, *Sep. Sci. Technol.*, 14 (1979) 935.
4 M. Martin and R. Reynaud, *22nd Eastern Analytical Symposium, New York, 16–18 Nov., 1983*, Abstract No. 96.
5 V. L. Kononenko and J. K. Shimkus, *J. Chromatogr.*, 520 (1990) 271.
6 Ph. J. P. Cardot and M. Martin, *1st International Symposium on Field-Flow Fractionation, Salt Lake City, UT, 15–16 June, 1989*, Abstract No. 23.
7 P. Jarolim, M. Lahav, L. Shih-Chun and J. Palek, *Blood*, 76 (1990) 2125.

8 J. C. Giddings, *Sep. Sci. Technol.*, 19 (1984) 831.
9 M. Martin, J. Gril, O. Besançon and R. Reynaud, *6th International Symposium on Column Liquid Chromatography, Philadelphia, PA, June 7–11, 1982*, Abstract FFF Session.
10 M. Martin, *12th Internal Symposium on Column Liquid Chromatography, Washington, DC, June 19–24, 1984*, Abstract TH-L.-24.
11 J. C. Giddings and M. N. Myers, *Sep. Sci. Technol.*, 13 (1978) 241.
12 B. A. Bidlingmeyer and F. V. Warren Jr., *Anal. Chem.*, 56 (1984) 1583A.
13 C. Sultan, G. Priolet, Y. Beuzard, R. Rosa and F. Rosso, *Techniques en Hématologie*, Flammarion, Paris, 1983.
14 W. J. Williams, E. Butler, A. J. Erslev and R. W. Rundles, *Hematology*, McGraw Hill, New York, 1972.
15 Ph. J. P. Cardot, *Thèse de Doctorat*, l'Université Pierre et Marie Curie, Paris, 1988.
16 A. Merino, C. Bories, J. Gantier and Ph. J. P. Cardot, *J. Chromatogr.*, 572 (1991) 291.
17 A. Merino, personal communication.
18 L. M. Corash, S. Piomelli, H. C. Chen, C. Seaman and E. Gross, *J. Clin. Med.*, 84 (1974) 147.
19 J. C. Giddings, M. H. Moom, P. S. Williams and M. N. Myers, *Anal. Chem.*, 63 (1991) 1366.
20 B. D. Hames, in D. Rickwood (Editor), *Centrifugation*, IRL Press, Washington, DC, 2nd ed., 1988, p. 45.
21 J. C. Giddings, M. N. Myers and K. D. Caldwell, *J. Chromatogr.*, 185 (1979) 261.
22 S. Lee, M. N. Myers and J. C. Giddings, *Anal. Chem.*, 61 (1989) 2439.
23 Ph. J. P. Cardot and M. Martin, *18th International Symposium on Chromatography, Amsterdam, June 18–23, 1990*, Abstract FR-P-014.
24 J. C.Giddings, *Polymer Mater Sci. Eng.*, 59 (1988) 156.