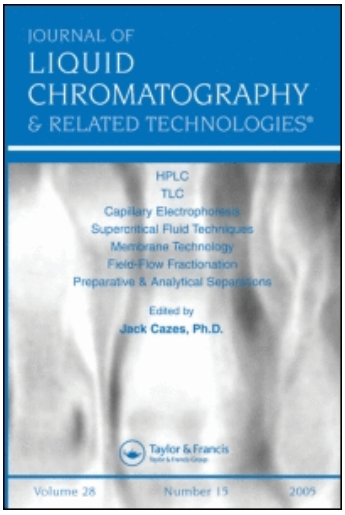

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AGE-DEPENDENT ELUTION OF HUMAN RED BLOOD CELLS IN GRAVITATIONAL FIELD-FLOW FRACTIONATION

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ABSTRACT

The technique of field-flow fractionation (FFF) which combines the earth gravitational force with a carrier liquid flow in a horizontal, ribbon-like channel is well suited for the separation of micron-sized particulate species such as cells. We investigated the selective elution, in a phosphate buffer, of human red blood cells (RBC) which migrate along the FFF channel more slowly than the carrier.

Fractions of the channel effluent were collected and the activities of various intracellular enzymes, which either reveal the presence of white cells or are known to be related to cell age, were evaluated for each fraction. The hemoglobin sub-fraction composition was also determined.

From analysis of these biochemical determinations, nucleated cells and reticulocytes appeared to be eluted as unretained species while mature RBC form a well defined, retained peak. The steady variation of the activity of age-related enzymes within this peak demonstrates that RBC are separated according to age. These observations, linked to the fact that reticulocytes have a drastically different FFF behavior from RBC, reveal that particle shape and stiffness are, when combined with size and density, key biophysical factors controlling the retention of biological micron-sized particles in FFF.

INTRODUCTION

Field-Flow Fractionation (FFF) is a dynamic separation technique based on the differential elution of the sample constituents by a laminar flow in a flat, ribbon-like, channel according to their sensitivity to an external field applied in a direction perpendicular to that of the flow.[1] FFF applies to a wide variety of macromolecular and particulate species, but the elution mechanism of Brownian, submicron colloidal species differs greatly from that of super-micron particles.[2,3] For the latter, the interplay of steric interactions of the particles with the channel walls and of hydrodynamic lift forces of various origins[4-6] leads to the earlier elution of larger spherical particles.[2,3,7,8] The corresponding elution mechanism is only poorly understood, even for rigid spherical particles. Still, FFF was shown to apply successfully to micron-sized, non rigid particles as evidenced by the increasing number of reports devoted to the separation of cellular materials such as human red blood cells.[9-17]

In the present study, human blood cells were analyzed by the simplest FFF technique, gravitational FFF (GFFF), in which the primary field is the natural earth gravitational field. In order to gain some insight into the separation mechanism of these "soft" particles by FFF, as well as to further evaluate the potential of this technique for blood cell analysis, fractions collected from the channel effluent were subsequently analyzed by means of different erythrocyte and blood markers. They allow a more detailed characterization of the effluent than classical UV photometers generally used in FFF of red blood cells (RBC).

METHODS

The gravitational FFF channel was made from the void space obtained after cutting a 92 cm-long, 2 cm-wide rectangular band with tapered ends in a 175 μm thick Mylar sheet. This sheet was compressed between two horizontal mirror glass plates coated with silicone oil (Silbione, Rhône-Poulenc, Paris, France) to avoid interactions between the cells and the channel walls. The septum injector, as well as connecting tubes from injector to channel and from channel to detector and to fraction collector, were Teflon. The inlet and outlet connecting tubes perforated the upper and lower glass plates, respectively. The overall dead volume from injector to detector was 3.35 mL. The channel effluent was monitored by a Waters 440 (Milford, MA, USA) UV photometer operating at 313 nm. Isotonic (290-320 mOsm/L) phosphate buffer (NaCl 0.1 M, Na_2HPO_4 1.12 M, KH_2PO_4 0.2 M, pH = 7.20) was used for sample dilution and as carrier liquid. A carrier flow of 0.3 mL/min was delivered by an HPLC reciprocating piston pump (Model 110, Beckman, Berkeley, CA, USA).

All human blood samples were collected from the same donor with EDTA as anti-coagulant and diluted in the carrier liquid. 100 μL of an approximately 2-fold dilution of human peripheral red blood cells, containing few residual nucleated cells and platelets, were injected with a syringe through the septum injector.

When fractions of the channel effluent were collected, the detector was removed and replaced by a fraction collector (Model 201, Gilson, Villiers-le-Bel, France). Three consecutive runs were performed under identical experimental conditions. Fractions from each run collected during the same time interval were combined together in a pool. The pools were analyzed by means of the following erythrocytes and blood markers : pyruvate kinase (PK, EC 2.7.1.40) and glucose-6-phosphate-dehydrogenase (G6PD, EC 1.1.1.49) activities,[18-21] foetal hemoglobin (HbF) and glycosylated hemoglobin (HbA1C) composition[22] for RBC; myeloperoxidase (MPO),[23] and alkaline phosphatase (AP, EC 3.1.3.1)[24] activities for leukocytes and platelets.

RESULTS

A typical fractogram of such a blood sample is presented in Figure 1. Three peaks were obtained. The third one corresponds to the RBC, as evidenced by the direct microscopic examination of the cells collected from this peak, which shows an absence of nucleated cells.

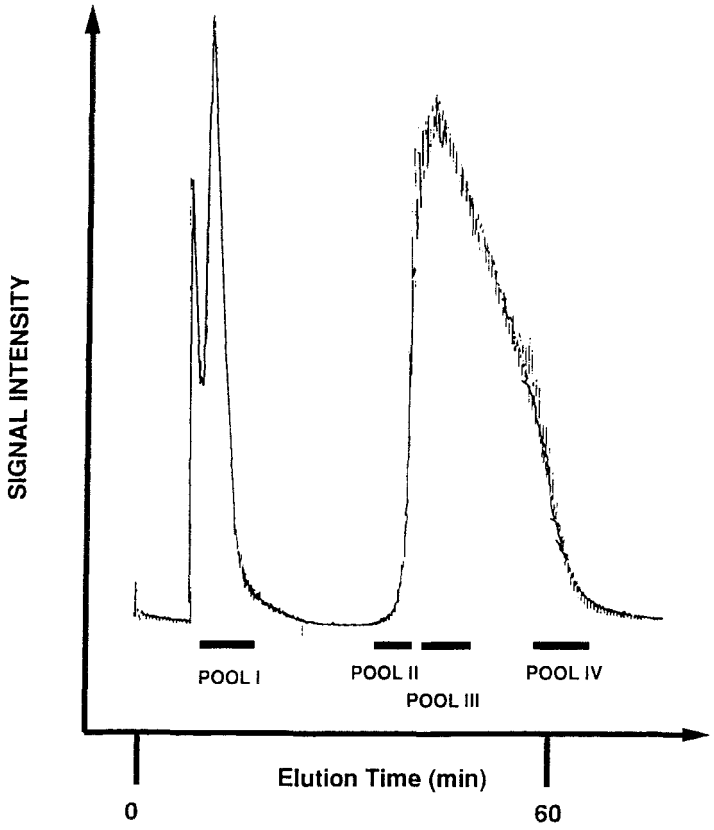

Figure 1. Fractogram of a human blood sample. The positions of the four collected fractions are indicated on the fractogram. Three successive runs were performed in identical experimental conditions. Fractions from each run collected during the same time interval were combined together in a pool. The enzymatic activities and hemoglobin compositions of the four pools so obtained were subsequently evaluated.

The first and second peaks correspond to particles and macromolecules like plasma proteins not affected enough by the external field to be retained. Four pools (see Figure 1) corresponding to the void volume peak (pool I), and to the beginning, the center and the end of the RBC peak (pools II, III and IV, respectively) have been selected for subsequent biochemical analysis. The results of the determinations are reported in Table 1. PK and G6PD activities clearly increase with the elution time, *i.e.*, from pool I to pool IV. MPO and AP activities observed in pool I indicate the presence of leukocytes, which could

Table 1

Biochemical Probes Characterizing the Four Pools Collected From the GFFF Channel Effluent*

Pool	Alkaline Phosphatase Activity	Myeloperoxydase Activity	Hemoglobin F Fract. (%)	Hemoglobin A1C Fraction (%)	Hemoglobin (Quantity Per Pool (mg)	Pyruvate Kinase Activity	Glucose-6-phosphate-dehydrogenase Activity
	0.81	1.43				1.0	0.3
I	0.74	1.35	1	6.2	0.27	0.8	0.1
						1.1	<0.05
II	<0.1	<0.15	0.2	8.5	2.49	6.8	4.3
						5.1	3.7
						12.3	6.5
III	<0.1	<0.15	0.4	8	25.84	13.4	5.6
						12.8	4.9
						21.3	10.3
IV	<0.1	<0.15	0.3	6.3	3.86	19.6	8.7
						18.2	9.4

* All enzyme activities were determined by microfluorometric methods referenced in text and are expressed as μmol/min/g hemoglobin. All assays were performed as recommended by the International Committee for Standardization in Haematology, except the final fluorometric measurements because of their low enzymatic activity levels.

explain the residual PK and G6PD activities in pool I. However, a small, but finite, hemoglobin content, suggesting the presence of a small amount of RBC or RBC lysates, was found in this pool. The subsequent analysis of hemoglobin profile revealed both a high percentage of hemoglobin F, as well as a high HbF/HbA1C ratio. These two values, which are quite larger than in pools II, III or IV, are of the same order of magnitude as that found in patients with hemolytic regenerative anemia and whose reticulocyte percentages number around 8%. This series of informations suggests that the few physiological circulating reticulocytes eluted within the fractions collected in pool I.

The microscopic observation performed on fractions of pools II to IV did not reveal the presence of nucleated cells, a fact corroborated by the undetectable activity of either MPO or PA. The significant increase of PK and G6PD activities observed from pool I to pools II-IV, therefore, indicates the presence of RBC in the latter, which was also checked by microscopy. Furthermore, PK and G6PD activities are seen to steadily increase from pool II

to pool IV, by a factor 2.5 for G6PD and larger than 3 for PK. These observations indicate that young cells, which have larger PK and G6PD enzymatic activities,[25] are retained longer than old cells. The hemoglobin subfraction analysis confirms this effect as the proportion of HbA1C, which is lower in younger cells,[26] decreases from pool II to pool IV.

The observed order of elution of RBC vs. age is in agreement with a previous report on the GFFF separation of transfused and endogenous RBC.[10] Using the quantitative correlation established by Corash, Piomelli et al.,[19] one can associate a RBC age to each value of the PK or G6PD enzymatic activity. Averaging the numbers obtained for each enzyme and each activity determination for a given pool, one gets the following means and standard deviations : 99 ± 19 days for pool II, 59 ± 20 days for pool III and 19 ± 6 days for pool IV. These figures, which have to be compared to the average lifetime of human erythrocytes in the circulation (120 days), reflect the high age selectivity obtained in a GFFF run of RBC. Compared to the classical density gradient centrifugation techniques,[19,25] GFFF offers a number of specific advantages : (i) a simple fractionation instrument, easily home-made, is used; (ii) the separation is more rapid than in density gradient centrifugation methods (in Figure 1, the run duration was about 75 minutes, however it has been demonstrated that the use of stronger driving forces such as a centrifugal force[9] or that force given by a cross-flow[11,14] instead of the earth's gravitational force, allows to obtain much faster separations); (iii) a small sample volume is required (about 0.3 ml of donor's blood have been used in the experiments); (iv) most importantly, the carrier liquid is kept unchanged during the duration of the FFF run, and its composition may be adjusted to physiological conditions (including sterilization) in such a way that the integrity of the cells is maintained, so that viable RBC can be collected at the end of the FFF channel for further use. The physiological constancy of the environmental medium confers a specific advantage to FFF over density gradient techniques for which the change of the osmolarity of the medium associated with the formation of the density gradient has been shown to induce volume and density changes to the RBC.[27]

DISCUSSION

It is tempting to interpret the retention behavior observed for RBC to that known for rigid spherical particles. However, the computation, from fluid mechanics principles, of the transversal lift force acting on particles in flow is not yet completed, even for rigid spheres, when the particles travel in the vicinity of a wall as it is the case in the FFF channel. Therefore, the

interpretation of RBC retention can only be qualitative. According to FFF retention models of micron-sized particles in which hydrodynamic lift forces play a dominant role,[4-6] the retention time of rigid spherical particles increases with decreasing particle volume and increasing particle density (when this density is larger than the carrier liquid density), as experimentally demonstrated.[3,7,8] It has been established that, as the RBC become older, their density increases,[27-29] their volume decreases,[27,28] and they become markedly less biconcave and more spherical.[28] One can, therefore, conclude that, since old RBC elute before young ones, the retention time of RBC increases with increasing cell volume, decreasing density and decreasing sphericity. Thus, as concerns the variation of retention with particle volume and density, the behavior of RBC appears to be opposite to the known behavior of rigid micron-sized spherical particles.

Still, the overall elution order observed between the leukocytes and the erythrocytes is in agreement with that of rigid spheres, since leukocytes are generally less dense and are bigger than RBC. Therefore, one is led to conclude that erythrocytes possess a specific physical characteristic which varies within the RBC elution peak and which influences their retention strongly enough to compensate the opposite combined effects of variations of volume and density. On a physical ground, the major differences between RBC and rigid spheres arise from their shape and the fact that RBC are extremely soft and elastic and may change their shape to a bell-like or paraboloid form when flowing through capillaries.[30] In addition, the orientation of these particles relative to the direction of the flow and the variations of the orientation induced by the velocity gradient may have some influence on the migration of the RBC. Lift forces appear, then, to be quite sensitive to the shape and the deformability of the particles. Because, as they become older, RBC become stiffer and their shape is less biconcave and more spherical, these findings suggest that the lift force increases with increasing sphericity and increasing stiffness of the RBC, if one assumes, in agreement with observations that the mean hemoglobin content per cell remains constant,[21,27,28] that the field force (weight of a RBC) does not change with cell age.

Intriguing is the observation, discussed above, that reticulocytes, which are the youngest RBC and become completely mature within one or two days after entering the circulation, are eluted in pool I, well ahead of the RBC peak, while young adult RBC are the more retained erythrocytes. Such a drastic behavioral difference has to be associated with a significant change in the biophysical characteristics during the maturation process of reticulocytes. It is known that these cells have a larger volume than mature RBC[31] and, from density gradient sedimentation experiments, that their density is smaller than

the average density of the RBC.[27] Although, as mentioned above, these effects can explain, according to known lift force models for rigid spheres, an earlier elution of the reticulocytes, they are unlikely to be the sole and, even, the dominating factors accounting for the retention difference between reticulocytes and normal erythrocytes since they cannot explain the relative retention of young and old RBC. One is led to speculate again on the major role played by shape and stiffness factors in the migration of the reticulocytes in the FFF channel. These factors might be associated with residual organelles[30] or morphological characteristics: reticulocytes appear to be more globular than RBC, although their shape is irregular and polylobulated.[32] Only in the late stage of their maturation process does the biconcave disk shape of the young RBC appear.[30]

The biochemical analysis of fractions collected from the FFF channel effluent has allowed to demonstrate that RBC are separated according to their age. In addition, it has revealed a complex and rich variety of retention behavior which suggests that particle shape and deformability play a dominant role in the flow of RBC. It is hoped that fluid mechanics difficulties involved in the calculation of the lift force on particles moving near a wall will be soon overcome. This would be of great interest for the quantitative interpretation of FFF retention data as well for a better biophysical understanding of blood circulation in capillaries.

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