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Comparative study of human red blood cell analysis with three different field-flow fractionation systems

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Abstract

An extensive multi-laboratory study was conducted to compare three different field-flow fractionation (FFF) systems for use in the analysis of human erythrocytes. The object of this study was to determine the relationship between the FFF elution properties for each system and the traditional hematological blood cell parameters. One centrifugal system (Utah) and two gravitational systems (Paris and Abbott) were compared. In order to analyze erythrocyte populations with a broad range of hematological indices, blood samples were collected from individuals heterozygous for sickle cell anemia (A/S) and also from normal controls (A/A), and these were analyzed at each site. Identical samples were analyzed by the Abbott and Utah sites. With all three systems, blood samples from each category produced narrow, overlapping distributions of FFF retention ratios, with the Abbott and Utah systems showing slight elevations in the mean retention ratios for the sickle cell samples. Blood cell elution peak characteristics were compared with standard hematological parameters for each of the FFF systems, and negative correlations were consistently found between mean corpuscular volume (MCV) and retention ratios. Positive correlations were found between red cell distribution width (RDW) and retention ratios. Elevated FFF retention ratios were frequently found with blood samples having abnormal hematological profiles. These results demonstrate that the three differently configured systems all produce similar analysis profiles for erythrocytes from the classes studied here. The relationships between FFF parameters and hematological indices were consistent for all systems.

Keywords: Red blood cells; Field-flow fractionation

1. Introduction

Field-flow fractionation (FFF) is a technology which can be used to separate cells or particles based on their size, density, and other physical characteristics [1,2]. Following injection of a sample containing particles or cells with different physical properties into an FFF channel, one observes the differential elution of the individual subsets of particles in an order dependent on their unique physical properties. There have been

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many reports regarding the use of FFF technology [3–10] for analysis of blood cells. Due to the unique parameters by which FFF technology fractionates, this offers a new approach for separating and analyzing cells which may prove to have important biological value. Andreux et al. [3] demonstrated that FFF analysis was able to resolve both reticulocytes, and Heinz body rich cells from normal erythrocytes with experimentally induced anemia in rabbits, showing that biologically important cell populations can be physically separated and monitored with FFF technology.

Although previous studies have shown that FFF technology can physically separate distinct cell populations, it has not been demonstrated which specific cell parameters are dominant in effecting this separation. Initial comparative studies of erythrocytes from different animal species [4,6] provided data indicating that cell size was a primary factor, consistent with FFF theory and previous work with synthetic microparticles. More recent studies [7,9], using human erythrocytes from different individuals, failed to find any correlation between the MCV of the blood samples and FFF elution position, indicating that other cellular properties, which vary between individuals, must also have a significant influence on FFF mobility. Cell density and membrane rigidity have both been implicated as important cell features which effect FFF elution order [7–9]. These cell parameters are not easily measured with current analytical instrumentation.

Various differences exist also in the configurations of the FFF instrumentation between the different laboratories working in this area, particularly with regard to channel size, field strength, flow speeds, and construction materials. There have been no comparative studies performed to elucidate whether the apparent discrepancies reported regarding the correlation between cell size and FFF elution position was due to differences in the cell samples analyzed or to different characteristics of the FFF systems. This study was undertaken to directly compare three different FFF systems and to obtain information about the correlation between FFF elution characteristics and physiological cell parameters.

2. Experimental

2.1. Reagents

Pre-made phosphate-buffered saline (PBS) packets and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA).

2.2. Human blood samples

Blood specimens from normal individuals and individuals with heterozygous (A/S) sickle cell anemia were obtained from local hospitals or the Interstate Blood Bank of Florida (Miami, FL, USA). Blood for the Paris studies was collected from sickle cell patients without any associated thalassemia disorders; whereas, samples for the Abbott and Utah studies were collected from patients independent of associated thalassemia. All specimen were collected in tubes containing EDTA as the anti-coagulant and were refrigerated until the time of analysis. In order to insure comparability between samples collected and analyzed at different sites, all of the samples were evaluated within one to two days after the blood was collected.

2.3. Hematology analyses

All routine hematology analyses for samples used by the Abbott and Utah laboratories were performed on the CELL-DYN 1600 CS Hematology Analyzer (Abbott Laboratories, N. Chicago, IL, USA) and samples used by the Paris laboratory were analyzed with an Argos Analyzer (Roche Diagnostic Systems, Branchburg, NJ, USA). Specimen were analyzed as soon as they were received.

2.4. Field flow systems

The centrifugal FFF system from the University of Utah and the gravitational system from Abbott have been characterized, and the reproducibilities in both systems were less than ±2% [4,9]. The gravitational system from the University of Paris was constructed as described by Cardot et al [5] using a pair of polycarbonate plates (Lexan, 100×10×600 mm). The bottom plate was drilled to glue inlet and outlet

tubings (0.256 mm I.D. 220 mm long PEEK, Upchurch Tubes) to its surface. An area (20×500 mm) with tapered ends was cut in a Teflon sheet (100×0.125×600 mm) and sandwiched between the two polycarbonate plates to form the completed channel.

A Linear UV-106 (Linear Instruments, Reno, NV, USA) detector operating with a 254 nm light source was used in Utah system, and a Knauer variable-wavelength detector (Cunow, Cergy, France) set at either 254 or 313 nm was used with the Paris system. Direct cell counts were made with the Abbott system using a CCD camera [9].

All systems were equilibrated with PBS containing 0.1% BSA and 0.01% sodium azide. Table 1 presents a summary of the configurations of the three systems.

2.5. Field-flow fractionation procedure

For analysis, blood samples were diluted with PBS containing 0.1% BSA and 0.01% sodium azide, to a final cellular concentration of $30 \cdot 10^6$ cells/ml. This dilution was performed no more than 10 min prior to injection into the FFF system. Analysis in each laboratory was performed under operating conditions which had been previously found optimal for that given system. The liquid eluted from the FFF channels during each analysis was collected and weighed to verify pump speed. Peak elution positions, initially determined as elution times, where converted to elution volumes using the experimentally determined channel flow-rate. Retention ratios (R) were calculated by dividing the channel void volume by the peak elution volume. Plate height (H) data were used to determine the relative widths of the elution peaks. For this purpose, H-values were determined graphically from peak widths at half heights [11].

2.6. Statistical analyses

All data are expressed as mean±S.D.. Statistical significance was determined by the Student's t-test and regression analysis using Sigmastat (Jandel Corp. v1.01, San Rafel, CA, USA).

3. Results

This study was initiated to compare the performance of three different sedimentation FFF systems for the analysis of human erythrocytes under controlled conditions. The systems evaluated were: (1) the centrifugal FFF system from the University of Utah [4], (2) the gravitational FFF system from the University of Paris, and (3) a miniaturized gravitational FFF system from Abbott Laboratories [9]. Table 1 lists the properties and configurations of each of these systems. A protocol was developed to minimize variables in testing such as sample collec-

Table 1
Configurations of the three FFF systems evaluated in this study

Parameter	Utah	Abbott	Paris
Field strength	4G	1G	1G
Channel dimensions			
Length (cm)	94.5	19.0	49.5
Width (cm)	2.0	0.85	2.0
Depth (μm)	250	125	125
Fluid flow velocity (cm/min)	60	7.9	6.4
Wall shear rate (s^{-1})	230	63	51
Injection position	Top	Top	Bottom
Relaxation	Stop flow (5 min)	Slow inject (4 min at 0.47 cm/min)	None
Accum. wall material	Hastelloy C	Glass	Lexan
Detector	UV (254 nm)	CCD camera	UV (254 nm)

tion and handling procedures, sample dilution and elution buffers, and data analysis procedures. Each laboratory analyzed a panel of approximately twenty normal blood samples, and samples from approximately twenty individuals, heterozygous for sickle cell anemia. The Utah and Abbott laboratories both analyzed the same samples to permit a direct system-to-system comparison. A common panel of fixed red blood cells was also analyzed by all three systems. The reproducibility of the systems were also tested, and found to be ±2% for the Utah and Abbott [9] systems

Diluted blood samples were analyzed, as described in Section 2.3 by all three laboratories, and in each case a broad single peak of eluted cells was detected (Fig. 1). Small void volume peaks (plasma proteins, platelets, etc.) were seen with the Utah (approx. 2 min) and Paris systems (approx. 7 min). This peak was not present with the Abbott system because it detects whole cells only. Because the three FFF systems had distinctly different field strengths, channel geometries, and fluid flow velocities, the elution positions of the erythrocytes and duracytes varied with each system. The resolution between the erythrocyte and duracyte peaks was greatest with the Utah system, and least with the Paris system, confirming that the physical configurations of the systems do significantly effect their resolving characteristics. Peak elution times were measured for each sample and then retention ratios were calculated as described in Section 2.5. The retention ratios, therefore reflect the mobility of the erythrocyte populations as they flow through the FFF channels. To determine whether peak skewing for some samples would affect the calculations, retention values were determined at both the peak maximum position and also at the peak center of mass position. As shown in Fig. 2, no significant differences were noted between the two retention ratios (R) (correlation coefficient r=0.968 and 0.946 for the Abbott and Utah data respectively), so all retention ratios presented in this report will be those determined at the peak position unless otherwise noted.

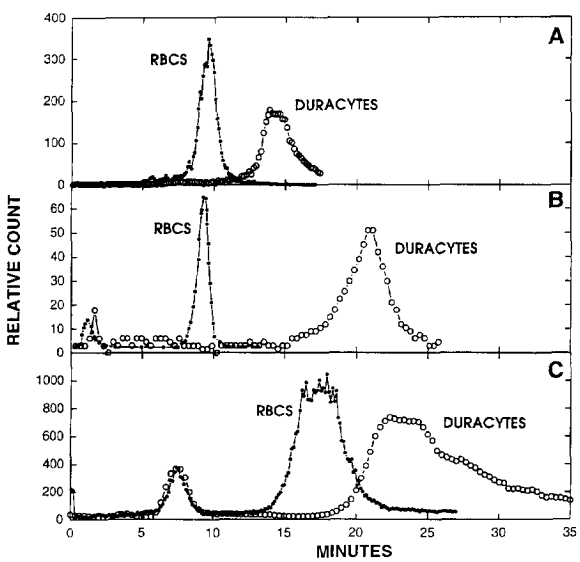

Fig. 1. Fractograms of normal blood (●), and fixed cells (duracytes) (○), from each different system run at optimal flow-rates. (A) Abbott; (B) University of Utah; (C) University of Paris.

Fig. 3 displays the retention ratios for all of the samples analyzed in this study. Average retention ratios of 0.216 (±0.010), 0.167 (±0.013), and 0.441 (±0.059) were found for samples from normal individuals from the Abbott, Utah, and Paris groups respectively. All testing centers found that retention ratios from normal blood samples clustered into a fairly tight region, specific for their FFF system; however, there was a single sample from each testing site with a retention ratio greater than 2 S.D. above the mean. For all systems, the population of retention ratios from the sickle cell patients was also a cluster which overlapped significantly with the normal population [R=0.220 (±0.011), 0.176 (±0.016), and 0.420 (±0.024), for the Abbott, Utah, and Paris groups, respectively]. The mean retention ratio for the sickle cell samples from the Utah data was significantly different from the normal mean (statistical p-value, calculated as described in Section 2.5, p=0.018); however, the other two systems did not show significant differences between the two means (p>0.05). In attempt to simulate cells with extremely stiff, rigid membranes, two types of fixed erythrocytes were analyzed with all three systems. With each FFF system, the fixed cells displayed markedly decreased retention times, ranging from 50% to 76% of the retention times found for normal unfixed cells.

The direct comparison between retention ratios of the Utah and Abbott systems on the same samples is shown in Fig. 4. The correlation coefficients between

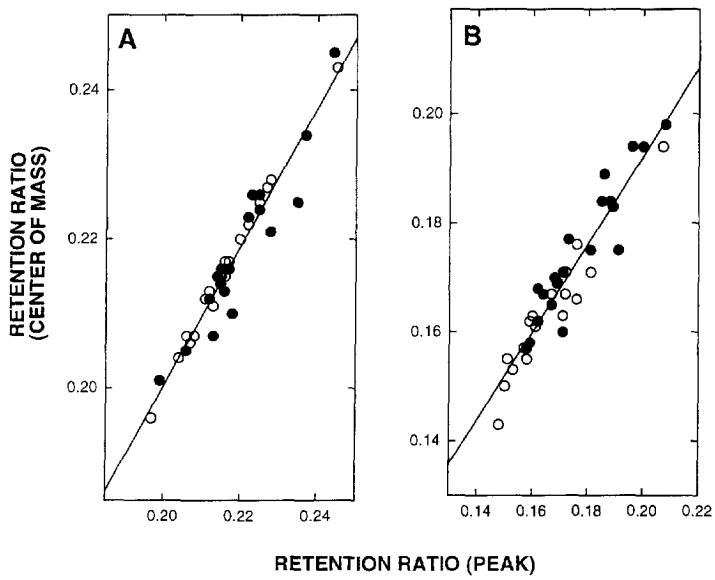

Fig. 2. Correlation between FFF retention ratios R calculated at the peak maxima or at the center of mass. (A) Abbott; (B) University of Utah. (○) Normal blood samples; (●) heterozygous sickle cell anemia blood samples.

the two measurements was 0.836 for all samples and 0.699 and 0.408 for the normal and sickle cell samples respectively. Despite the major differences in the configurations of the Utah and Abbott systems (field force, flow velocity, channel depth, etc.), it is notable that such a degree of correlation existed between the two systems.

To characterize the influences of lift forces on

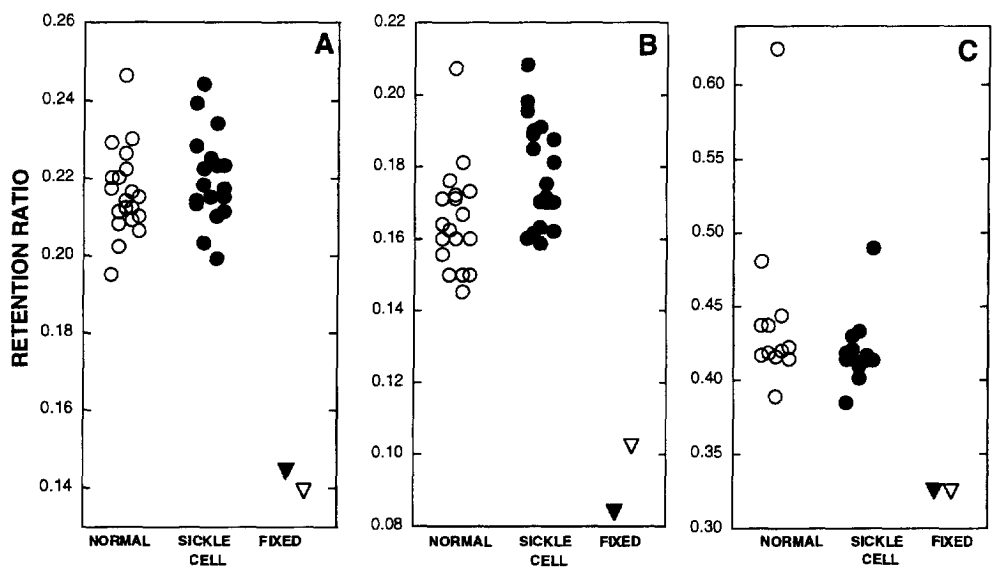

Fig. 3. FFF retention ratios R for blood samples and fixed cells. (A) Abbott; (B) University of Utah; (C) University of Paris. (○) Normal blood samples; (●) heterozygous sickle cell anemia blood samples; (▽) glutaraldehyde fixed erythrocytes; (▼) duracytes.

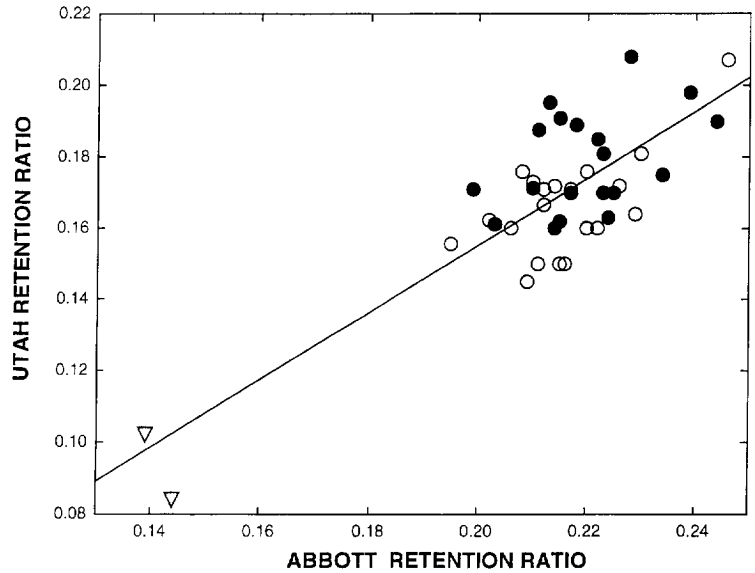

Fig. 4. Correlation between FFF retention ratios R determined at Abbott and the University of Utah on the same samples (r=0.836). Reproducibility in both systems were determined to be less than ±2%. (○) Normal blood samples; (●) heterozygous sickle cell anemia blood samples; (▽) fixed erythrocytes.

erythrocyte mobility in the FFF systems flow rate studies were performed with both normal and sickle cell samples. The retention behavior of fresh and fixed red blood cells was compared at different flow-rates for all systems. In case of Utah system, with its variable field strength, this parameter was systematically varied as well. Optimal conditions were considered to be those yielding maximal difference in retention. Flow-rates of 50%, 75%, 100%, 150%, and 200% of the optimal values for each FFF system were run, and Fig. 5 displays the data from flow-rate studies with normal and sickle cell samples from each testing site. For each system, retention ratios measured with most samples (both normal and sickle) showed a very similar response to changes in flow-rate, indicating that the lift forces are very similar for cells from each of the samples. The panel of samples used for the Abbott and Utah studies included ones with mean cell volumes (MCVs) ranging from 49 to 90 fl and the Paris set included samples with MCVs ranging from 73 to 91 fl, and most displayed nearly identical flow-rate response curves. The slopes and magnitudes of the retention ratios for the Abbott and Utah systems were again very similar. One sickle cell sample (MCV=49 fl) showed a significantly steeper slope as compared to the other samples with the Utah system. This same sample showed a normal retention slope with the Abbott system. The Paris system had higher retention ratios with greater sensitivity to changes in flow-rate. Two types of fixed cells were also run in flow-rate studies with all three systems (Fig. 6), and these showed similar patterns to the fresh cells, but at lower overall retention ratios and with less sensitivity to flow-rate changes.

Table 2 shows the correlation coefficients between the FFF retention ratios and the standard hematological indices for the erythrocytes in the blood samples analyzed. Correlation coefficients which were greater than 0.5 or less than −0.5 are highlighted. Similar patterns were found for all three FFF systems. Correlation coefficients with the highest degrees of significance were found most commonly in the relationships between R values and either MCV or RDW (Fig. 7). Negative correlations were always seen between the blood sample MCV and the FFF retention ratios. A positive correlation (frequently greater than 0.6) was consistently seen

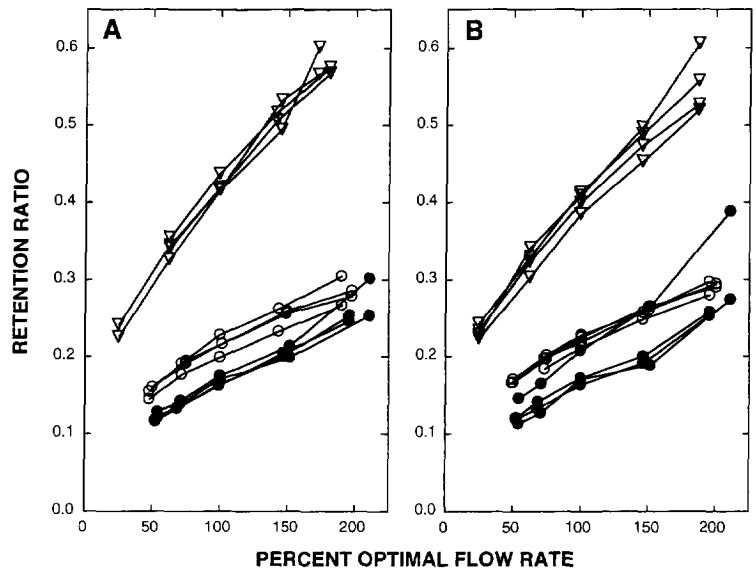

Fig. 5. Effects of flow-rate on FFF retention ratios R for blood samples from normal individuals (A) and heterozygous sickle cell anemia patients (B) determined at three different study sites. (○) Abbott; (●) University of Utah; (▽) University of Paris.

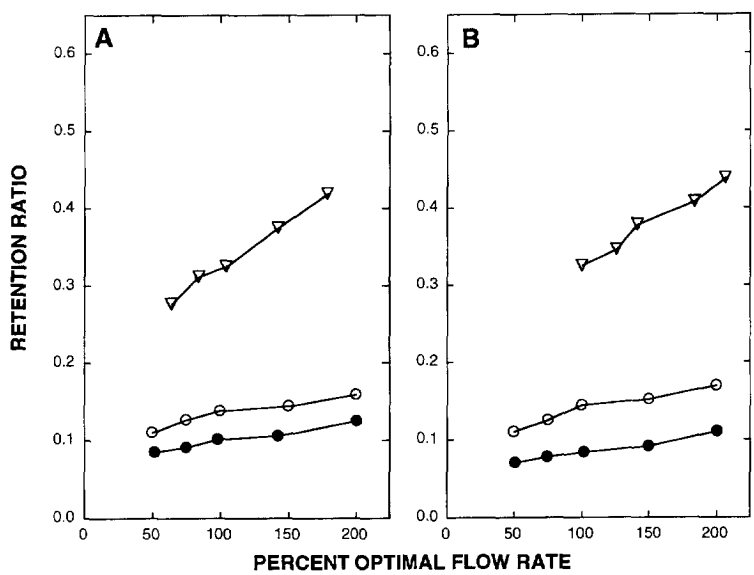

Fig. 6. Effects of flow-rate on FFF retention ratios R for glutaraldehyde fixed erythrocytes (A) and duracytes (B) determined at three different study sites. (○) Abbott; (●) University of Utah; (▽) University of Paris.

Table 2
Correlation between the FFF retention ratios R, and the Hematological Indices

	MCV	MCHC	RDW	RBC
Abbott				
Total	−0.311	−0.366*	0.423*	0.180
Normal	−0.322	−0.322	**0.598***	0.217
Sickle	−0.257	−0.390	0.261	0.085
Utah				
Total	**−0.683***	−0.197	**0.702***	0.261
Normal	**−0.521***	−0.015	**0.773***	−0.022
Sickle	**−0.771***	−0.303	**0.579***	0.321
Paris				
Total	**−0.577***	−0.093	0.247	0.269
Normal	**−0.828***	**−0.619***	**0.751***	0.319
Sickle	**−0.661***	0.446	0.474	0.328

The data is expressed as the correlation coefficient. MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; RBC, red blood cell count.
Bold numbers represent values greater than 0.5 or less than −0.5.
* Represent data points of greater significance ($p<0.05$); for all other data $p>0.05$.

between the FFF retention ratios and RDW values. This indicates that blood samples from this study, with more diverse cell size populations displayed higher retention ratios. Significant correlations between FFF retention ratios and mean cell hemoglobin content (MCHC) or red blood cell (RBC) counts were infrequently found.

Relative peak width values (H, calculated as described in Section 2.5 were determined to compare the elution peak profiles between the different samples and FFF systems. This value directly reflects both the position and width of an eluted peak, where broad, poorly retained peaks have high values and sharp, highly retained peaks have low values. Table 3 shows the correlation coefficients between FFF relative peak widths and the standard hematological indices for the erythrocytes in the blood samples studied. A similar pattern was seen for all three FFF systems. Once again, MCV and RDW are the two hematological indices with the most highly significant correlations. Fig. 8 shows direct comparisons between both MCV and RDW with respect to the relative peak widths for the samples analyzed in this study.

In these studies, elevated FFF retention ratios and elevated relative peak widths were most commonly found to be associated with low MCV indices or elevated RDW values. We next decided to look at the retention ratios in samples which had been grouped by hematological status, instead of hemoglobin types. Fig. 9 shows the relationship between re-

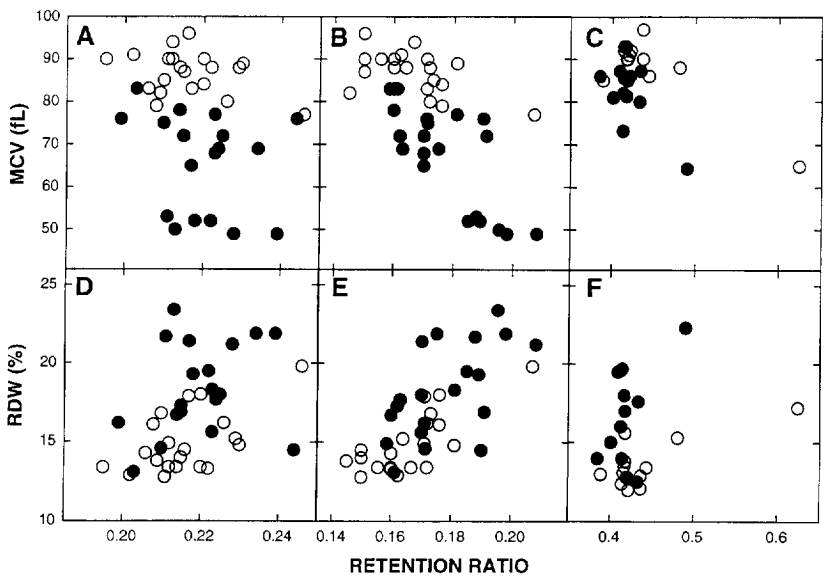

Fig. 7. Correlations between FFF retention ratios R and either MCV or RDW for the three different study sites. (A and D) Abbott; (B and E) University of Utah; (C and F) University of Paris. (○) Normal blood samples; (●) heterozygous sickle cell anemia blood samples.

Table 3
Correlation between the FFF relative peak width (H) values and the Hematological Indices

	MCV	MCHC	RDW	RBC
Abbott				
Total	**−0.558***	−0.309	**0.665***	0.260
Normal	−0.327	0.225	0.406	−0.296
Sickle	**−0.667***	**−0.758***	**0.763***	0.372
Utah				
Total	**−0.656***	−0.451*	**0.707***	0.293
Normal	0.140	**−0.670***	−0.171	0.134
Sickle	**−0.801***	**−0.572**	**0.790***	0.200
Paris				
Total	**−0.667***	−0.051	0.318	0.424*
Normal	**−0.865***	**−0.522**	**0.705***	**0.518**
Sickle	**−0.645***	0.242	0.391	0.406

The data is expressed as the correlation coefficient. MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; RBC, red blood cell count.
Bold numbers represent values greater than 0.5 or less than -0.5.
* Represent data points of greater significance ($p<0.05$); for all other data $p>0.05$.

tention ratios and hematological status for the samples analyzed by the Abbott and Utah groups. For both sites, the groups of samples with low MCV and high RDW showed elevated mean values as compared to samples with all normal hematological indices ($p=0.020$ and <0.001 for the Abbott and Utah data, respectively).

4. Discussion

A primary intent of this study was to compare three differently configured FFF systems which have been used for the analysis of erythrocytes from human blood. In general, all three systems revealed a very similar pattern of performance although the absolute values of the retention ratios were different for each system. The Utah and Abbott systems showed the closest correlation despite the major differences in field strength, fluid flow-rates, and channel sizes. The Paris system showed much higher retention ratios (weaker retention) compared to the other systems. This is surprising, because the configuration of the Paris system is very similar to the Abbott system, but may be explained by differences in channel surface composition, given that the Abbott system forces the cells to accumulate against a glass plate, while the Paris version involves accumulation at a Lexan surface. Another possible explanation might be the lack of relaxation of samples after injection in Paris system. Results from these studies

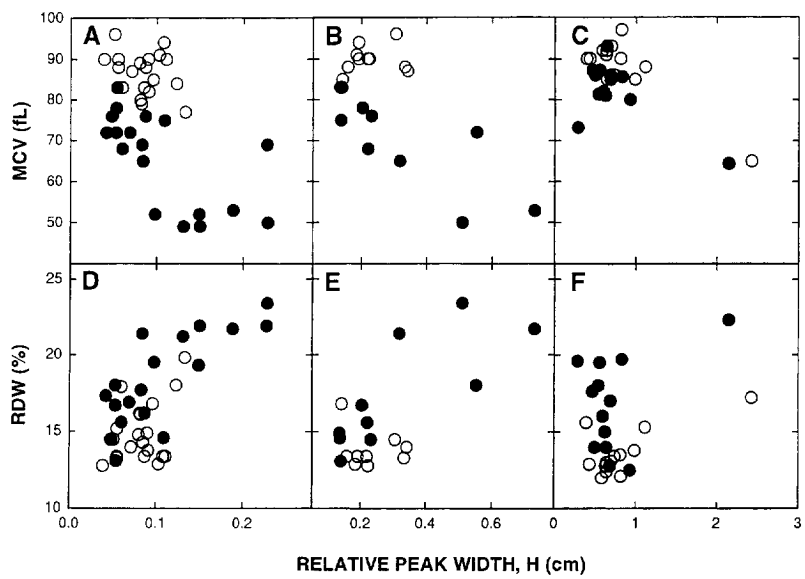

Fig. 8. Correlations between FFF relative peak widths (H) and either MCV or RDW for the three different study sites. (A and D) Abbott; (B and E) University of Utah; (C and F) University of Paris. (○) Normal blood samples; (●) heterozygous sickle cell anemia blood samples.

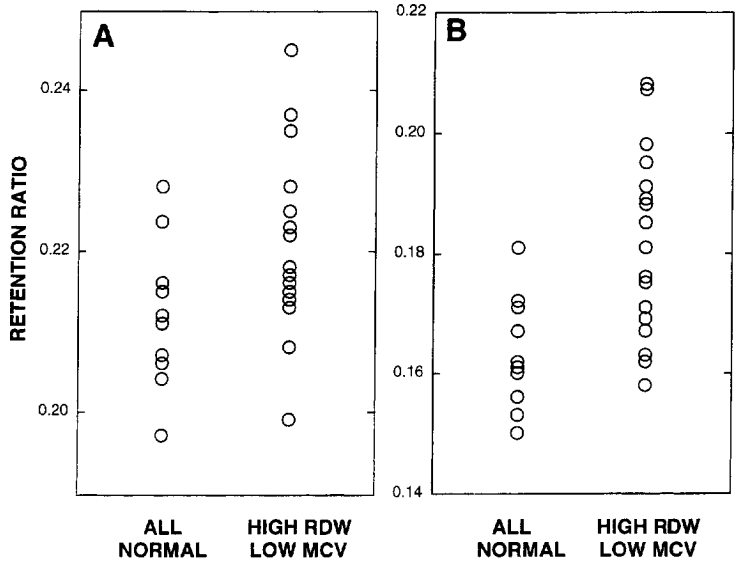

Fig. 9. Relationship between FFF retention ratio R and hematological status grouping (MCV and RDW) for two study sites. (A) Abbott; (B) University of Utah.

suggest that other factors such as sample injection position, relaxation processes, and accumulation wall materials may be very important in defining the ultimate performance of FFF systems for erythrocyte.

FFF retention ratios from the populations of blood samples analyzed in this study, showed a very narrow range of variation for each system. Occasional samples with elevated FFF retention ratios (>2 S.D. above the normal mean), were found from both the normal and sickle cell sample groups, however, no samples were found with retention ratios >2 S.D. below the normal means. A slight increase in the average retention ratios for the sickle cell samples was found with the Utah and Abbott systems. This is in agreement with previous reports [7,9] which also found that erythrocytes from sickle cell anemia patients and from placental cord blood flowed more rapidly through the FFF channel (increased retention ratios). Opposite to what has been seen with pathological blood samples, fixed and crosslinked human erythrocytes eluted from the FFF channels much later than fresh, normal cells, producing lower retention ratios. Chemical fixation of erythrocytes has been previously shown to increase membrane rigidity and we have also determined that it increases cell density (unpublished results). Increases in both density and rigidity are both factors that would be expected to retard cell flow and lower the retention ratios.

Early FFF studies with polystyrene microparticles or red blood cells from different animal species [1,4] indicated that fractionation by this technology was based primarily on cell or particle size, with the larger cells having greater mobility in the FFF channels. Results presented here, similar to two recent studies [7,9], failed to find this same correlation, when cell samples from many different individuals were analyzed. This indicates that when monitoring individuals of different hematological status, factors other than cell size, are important in determining the mobility of erythrocytes through the FFF channels. Since the study described in this report evaluated restricted populations of samples, many of which commonly had low MCV values, it is possible that the apparent negative correlation with MCV that we observed is indirect, and it is really reflecting some other cell properties.

The increased retention ratios found in some blood samples may be due to larger populations of recticulocytes and young erythrocytes in those samples. Using an experimental rabbit model, Andreux

et al. [3] showed that reticulocytes and newly regenerated erythrocytes had increased retention ratios as compared to normal cells. It has also been shown that low density human erythrocyte fractions, known to be enriched in reticulocytes and young erythrocytes [12], flow rapidly (increased retention ratios) through FFF channels [9]. We have observed increased numbers of cells in low density fractions of blood from heterozygous sickle cell patients (Yue and Parsons, unpublished observations). Roberts and El Badawi [13] found that the RDW was proportional to reticulocyte counts in blood samples from anemic patients, thus the correlation between RDW and FFF retention ratios demonstrated here, may also reflect the relationship between retention ratios and reticulocyte counts.

MCV and RDW have proven to be valuable and sensitive markers for red blood cell pathologies and Bessman et al [14] have based a classification system for anemias upon the use of MCV and RDW values. The most common abnormalities of hematological indices, observed in the pathological samples from this study, was low MCV and high RDW (75% of the sickle cell samples from the Abbott and Utah sites). FFF retention ratios directly correlated with these indices. In concordance with this, the samples from the normal populations, which had elevated FFF retention ratios (>2 S.D. above the mean), also had abnormally low MCVs and high RDWs. These samples may potentially have been from individuals with mild cases of iron or other metabolic deficiencies(conditions which may result in abnormal RDW and MCV indices) which were not identified when the samples were classified as "normal". The stratification of our sample populations into categories of MCV and RDW status shows that the retention ratios are significantly elevated in categories with abnormal MCV and RDW values. This relationship between MCV, RDW and FFF retention ratios may indicate that retention ratios could prove to be useful markers for monitoring erythrocyte regeneration in anemia cases. Further studies will be necessary to more fully establish the utility of using this technology.

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