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VELOCITY ALLOWED RED BLOOD CELL CLASSIFICATION

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Abstract: Normal red cell dynamic behavior could be important in circulatory function. It may initiate many cellular damages, particularly in adhesion reactions with blood vessel endothelium. The purpose of this study is to use velocity criteria to classify normal red blood cells (RBCs) under dynamic conditions. Three classification methods were used, K-Means, HAC and LAMDA. They pointed out two classes of red blood cells with a third one at the boundary between the two others. This particular composition may influence cell specific behavior, especially under pathological conditions. *Copyright © 2007 IFAC*

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1. INTRODUCTION

Red cells dynamic behavior represents an important phenomenon according to their physiologic role in blood circulation. In normal conditions, cell behavior initiates many cellular and tissular damages, particularly adhesion reactions, as seen in vascular diseases as sickle cell disease (SCD) and malaria (Eaton, *et al.*, 1976; Hebbel, *et al.*, 1980; Hoover, *et al.*, 1979; Kaul and Nagel, 1993; Kumar, *et al.*, 1996). In fact, some authors demonstrated that cell adhesion may be modulated in pathologic conditions such as inflammation, vascular disease and during different medical treatments (Eaton, *et al.*, 1976; Hebbel, *et al.*, 1980; Hoover, *et al.*, 1979; Kaul and Nagel, 1993; Kumar, *et al.*, 1996; Smolinski, *et al.*, 1995). As endothelial cells are subject to flow shear stress, it is important to determine the detailed velocity distribution in microvessels in the study of mechanical interactions between blood and endothelium.

The present study focuses on the normal red cell dynamic behavior especially RBC velocity using a flow chamber associated to an apparatus of video-microscope image analyzer. This method simulates blood flow conditions as in normal situation, and can be used to simulate pathologic conditions as in case of inflammation status, vascular diseases, where

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blood cells and endothelium interactions are modified. Three standard classification methods were used, usually, K-Means, HAC and LAMDA to determine RBC classes. The results allow us to establish a preliminary profile of RBC velocity in order to compare to profiles obtained after vascular disease analysis.

2. MATERIAL AND METHODS

2.1 Endothelial cells.

Transformed Human Bone Marrow Endothelial Cells (TrHBMEC) were kindly provided by Dr. Weksler and Pr. Elion. TrHBMEC were maintained in humidified air/5% CO_2 at 37°C. TrHBMEC monolayers were grown to confluence in gelatin-coated Thermanox slides (Nalgene Nunc International) as previously described by Schweitzer, *et al.* (1997).

2.2 Red blood cells

Peripheral venous blood samples were obtained from healthy normal volunteers (AA) at the Centre National France Transplant (Hôpital Saint-Louis, Paris). RBCs were isolated from whole blood by repeated centrifugation and washes with saline. They were suspended in endothelial cell culture medium, without fetal calf serum and adjusted to 10^6 red cells/ml for flow assay.

2.3 Flow chamber

The flow chamber is composed of a rectangular plexiglas cavity (0.2 mm height, 29 mm length, 5 mm width). The bottom wall of the chamber is a Thermanox coverslip (0.17 x 60 x 24 mm^3) where endothelial cells were first coated. RBCs in suspension were directly injected into the chamber by the use of a plastic syringe. An electric pump (Havard Apparatus) generates a controlled flow according to normal vessel flow, 1 dyne/cm^2, 10 minutes. The flow chamber is associated with an inverted microscope (Nikon Eclipse TE300, X20). The real-time images coming from the flow chamber are recorded with a video tape recorder (Sony time lapse 168) and analyzed with a specific video analyzer (Pentium with Matrox digitized card). The experimental data obtained as velocity, acceleration, angular deviation, linearity index are used to characterize red cells trajectories, cell adhesion and cell-cell interactions. All experiments were performed at room temperature and repeated three times.

2.4 Classification methods

Three standard classification methods were used:

- K-Means clustering
- HAC: Hierarchical Agglomerative Clustering
- LAMDA: Learning Algorithm for Multivariate Data Analysis

HAC and K-Means are classical methods whereas LAMDAis a recent research method. The two first one have been successfully used in the biomedical fields (Murase, *et al.*, 2001; Norcum, 1999).

K-Means is a classical method based on the evaluation of distances between the sample and the provided centers of classes. The latter is given by the user at the beginning of the classification. These centers are then modified during the classification.

Furthermore, HAC is a hierarchical method based on hierarchical level of aggregation. The method provides several level of classification which are included. The visualization of the classification is made with a binary tree.

LAMDA is based on an original idea of Aguilar-Martin, *et al.* (1980). It has been successfully used in pattern recognition (Piera-Carreté, *et al.*, 1990), in biomedical imaging (Chan, *et al.*, 1989), for system of wastewater treatment (Waissman-Vilanova, *et al.*, 2000) and for bioprocesses (Aguilar-Martin, *et al.*, 1999). It is based on the evaluation of a membership function which is a generalization on the interval [0,1] of a binomial law of {0,1}. The membership function is given by:

$$M(x)= \rho^{a(x)} . (1-\rho)^{(1-a(x))} \qquad (1)$$

Where $a(x) \in [0,1]$ is a presence function and $\rho \in [0, 1]$. a(x) is generally a distance computed between a center of class and the sample. ρ is given by the user. This method is detailed in the following publications (Regis, *et al.*, 2004a; Waissman-Vilanova , *et al.*, 2000a).

The three methods gave different class centers. Number of classes and its centers are fixed beforehand in K-Means method while these parameters depend only on level of classification chosen by the users in HAC method. In LAMDA method, number of classes and their centers are computed according to the initial database (Regis, *et al.*, 2004a; Regis, *et al.*, 2004b; Waissman-Vilanova, 2000b).

3. RESULTS

3.1 Trajectory analysis

The real-time images coming from the flow chamber are recorded and analyzed into numerical data.

This study is based on the hypothesis that RBC velocity distribution should be a Gaussian one, calculated using Unilog, Stat 2005 software. The velocity distribution is estimated by a standard histogram (fig 1). Each bar represents the percentage of cells presenting the same mean velocity. The

histogram obtained show local maximum, suggesting that the distribution is a mixture of two or three Gaussian distributions which confirms an heterogeneous RBC population. Such a mixture can be estimated by using the well-known MCEM algorithm which requires an a-priori number of components of the mixture, that is the number of probabilistic classes. Most of RBC velocities are concentrated around 880,60 µm/ms, as seen in figure 1. Two other groups are concentrated around 1071,11 µm/ms and 1261,62 µm/ms.

3.2 Class number

Different RBC subgroups are obtained using classification method as HAC, K-Means and LAMDA. The result of this classification is a distribution into two classes, as presented in table 1, indicating that RBC population is composed of two different classes, a slow one, class 1 (C1) and a faster one, class 2 (C2) according to mean velocity (MV). RCN represents red cell number.

Table 1 Velocity-class distribution of HAC, K-Means and LAMDA methods.

	K-Means		HAC		LAMDA	
	MV	RCN	MV	RCN	MV	RCN
C1	750,08	25	899,79	48	899.37	47
C2	1199,77	33	1515,44	10	1515,10	11

Indeed, with K-Means, the class centers for C1 and C2 are, respectively, 750,08 µm/ms for 25 cells and 1199,77 µm/ms for 33 cells. However, with HAC method, the class centers for C1 and C2 are, respectively, 899,79 µm/ms for 48 cells and 1515,44 µm/ms for 10 cells. Similar results are obtained with LAMDA: 899,37 µm/ms for 47 cells and 1515,10 µm/ms for 11 cells.

Cell distributions are presented in figure 2 for HAC and LAMDA and in figure 3 for K-Means. C1 repartition indicates that this class is more extended with HAC and LAMDA methods (499,58 to 1219,55 µm/ms, white triangles, fig 2) than with K-Means method (499,58 to 872,56 µm/ms, white triangles, fig 3). This distribution is different in C2 class. The values vary from 1302,27 to 1950,45 µm/ms (black squares, fig 2) with LAMDA and HAC. This interval is reduced when using K-Means. It vary from 901,59 to 1950,45 µm/ms (black squares, fig 3). Thus, HAC and LAMDA class repartition is more homogeneous for C1 class. On the contrary, K-means makes C2 more homogeneous. In parallel, RBC are better distributed into K-means clusters (C1 RCN = 25; C2 = 33) than with HAC and LAMDA methods (C1 RCN = 47; C2 RCN = 11).

For the first time, we can establish a velocity profile for a normal red cell population. We obtained two classes with different RCN and MV (table 1). The largest class is the slowest with HAC and LAMDA (fig 2). On the contrary, the largest class is the fastest with K-Means (fig 3).

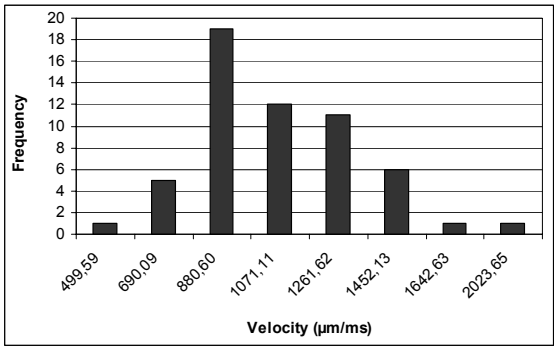

Fig. 1. Statistical distribution histogram of normal RBC velocity. Frequencies are represented in Y-coordinate and velocity (µm/ms) in X-coordinate.

The difference observed between K-means, HAC and LAMDA methods could be explained by the existence of a third medium class at the boundary between the two others.

4. DISCUSSION – CONCLUSION

In this paper, a chamber flow was used to simulate the blood flow and measure RBC velocity. The results show that the normal red cell population can be classified into two major groups, according to RBC velocity, a slow one, C1 and C2, with the highest mean velocity. It appears necessary to understand the biological importance of these classes. Indeed, this repartition could influence red cell biological behavior. In that case, red cells will have different role on circulatory function. Further studies are necessary to confirm those observations and determine biological effects of RBC velocity. In that purpose, other classification methods as decision

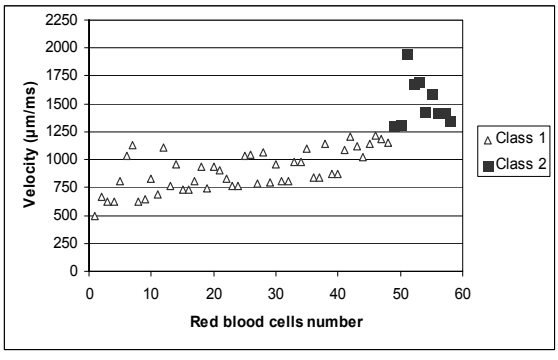

Fig. 2. HAC and LAMDA cell class repartition. C1 cells are presented by white triangles and C2, by black squares.Velocity of each red blood cell is represented in Y-coordinate (µm/ms) whereas each blood cell is represented in X-coordinate.

trees, expert system and biological experiments could be tested. Several studies were made on red cell velocity, to better understand the physiological effect, particularly in pathological conditions. Since

1971, Rosemblum (1971) observed a difference between red blood cell velocity and plasma velocity . He already noticed an heterogeneity in RBC velocity in venules (Rosemblum, 1971; Rosemblum, 1972a; Rosemblum, 1972b; Rosemblum, 1976). Our results confirm this heterogeneity observation. Bishop *et al.* (2001) suggested that RBC aggregation, defined as cells come into close proximity, may have a significant effect on circulatory function and on *in vivo* hemodynamics. These authors showed a different evolution according to the blood cell position, axial migration or near the wall of veins. This observation suggests a different red cell velocity distribution, confirmed by the present study. However, the experimental conditions used here do not allow us to define each class according to its location into the vessel. Even thought, we can hypothesize that the slowest class could be located near the endothelial cells wall and the fastest class, more in the axial migration. RBC trajectory studies (linearity, etc…) will be necessary.

However, the class composition could be explained by RBC characteristics such as cell age or density. Recently, Grima (2007) indicated that cell's velocity is primarily determined by the chemical gradient. Major factor in red cell compaction, particularly in haemoglobinopathies such as SCD, influence the cell characteristics, facilitating their adhesion. Further works are necessary to determine the biochemical, cellular, morphological and trajectory specificities, responsible for the dynamic differences observed. Embury et al. (1999) reported that the blood flow velocity in medium venules, in a mice model was about 1,1 ± 0,2 mm/s. This value is different from the in vitro model we used, where the mean velocity for the total population is 1,0 ± 0,30 mm/ms. This difference may be explained by in vitro experimental conditions, especially, the controlled blood flow and the lack of viscosity. They also pointed out blood flow abnormalities and detected decreased blood flow velocity in venules of all diameters in SCD mice model (Embury, et al., 1999; Paszty, et al., 1997). Indeed, in normal medium venules, the blood velocity is about 1,1 ± 0,2 mm/s compared to SCD medium venules were the blood velocity is less than 0,2 ± 0,1 mm/s (Embury, et al., 1999).

All these different experiments suggest that blood flow studies would be useful to understand vascular disease mechanisms. It will be interesting to apply cell classification method according to velocity, under various conditions as inflammation or medical treatment.

To conclude, these different studies may be useful to determine a new approach to quantify and characterize RBC dynamic behavior, according to the pathological state, as in medical treatment, cellular damages or vascular diseases.

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