Short Communications

Impaired Erythrocyte Doublet Formation in Diabetes

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Summary. A new means by which to examine erythrocyte deformability, the rate at which erythrocytes in dilute suspension form doublets after settling to the surface of a microscope slide, has been developed and tested. Doublet formation consists of the elevation and subsequent apposition of one cell over another, a process limited by the ability of each red cell membrane to bend. The cell-cell attraction that promotes doublet formation is controlled by adding an appropriate amount of dextran to the artificial suspending medium. Videotaping permits careful analysis of doublet formation rate and maintains a permanent record. When erythrocytes from 20 diabetic and 20 non-diabetic subjects were studied, doublet formation rates were found to be strikingly different. Cells from half of the diabetics studied formed less than three doublets in 20 min while the non-diabetic mean for the same period was 15 doublets. No overall correlation between doublet formation and fasting glucose could be found. No relation between doublet formation rate and type of diabetes, treatment, or microvascular complications was observed. Doublet formation rate is a simple and rapid means of detecting and studying reduced erythrocyte deformability in diabetes.

Key words: Diabetes, erythrocyte, haemorrheology, aggregation, microangiopathy, smoking.

ic erythrocytes based on studies using three- or five-micron polycarbonate sieves [2–7]. Diminished packing of centrifuged diabetic erythrocytes has also been interpreted as demonstrating reduced flexibility [8]. Two negative reports have also appeared. In one study, diabetic erythrocytes were moved at low velocity through three-micron capillaries without increased resistance [9]. In the other study, diabetic erythrocytes were shown to have a normal resistance to stretching and a normal rate of return to their initial shape [10].

Altered erythrocyte behaviour in diabetes may explain both the capillary basement membrane thickening found in the disorder and the clinically recognizable vascular changes associated with diabetes. The burden placed by rheologically abnormal diabetic erythrocytes on the circulation must be better understood in order to determine how the vessels are damaged. For this reason a new and independent means of testing erythrocyte deformability was sought. Doublet formation in dilute suspensions of erythrocytes has not previously been utilized in studying the effect of disease on erythrocyte deformability [11]. We have developed a technique for the standardized observation of doublet formation rate and found a striking difference between diabetic and non-diabetic erythrocytes; the technique and test results are described in this report.

Subjects and Methods

Twenty diabetic subjects (mean age 57 years, range 31–75 years) who were regularly attending an outpatient clinic were selected for study. History of onset and clinical course were used to classify each diabetic as Type 1 (insulin dependent) or Type 2 (insulin independent) and to establish duration of diabetes (mean 13.6 years, range 1–25 years). Each diabetic was also examined for evidence of microangiopathy based on the presence of microaneurysms, in-

In 1978 we reported an abnormally high resistance to flow when diabetic erythrocytes suspended in Tris Ringer solution were moved in a standard oscillatory pattern in four-micron glass micropipettes [1]. Diabetic red blood cells regularly required greater pressure than non-diabetic red cells for the same rapid motion. There are six reports of reduced filterability of diabet-

Fig. 1 a and b. Photographs of two videotape frames illustrate formation of an erythrocyte doublet. The numbers in white indicate the passage of time to be 9 s. Above the time is the frame count, which changes from one to sixty each second. **a** Just below and to the right of the centre of the picture (arrow), one erythrocyte has begun to climb over the erythrocyte directly above it. **b** A nearly completed doublet is seen (arrow). The entire slide surface seen by the videotape is approximately 120×155 microns and typically contains 100 erythrocytes

Fig. 2. The number of doublets formed is shown for all five erythrocyte preparations from each of 20 diabetic and non-diabetic subjects. Each preparation was observed for 4 min. A threefold mean difference is seen; $t = 5.9$, $p < 0.0001$. The majority of diabetic doublet formation rates are below the lowest non-diabetic rate

Fig. 3. Diabetic erythrocyte doublet formation rate is plotted against fasting plasma glucose level. The two diabetics with normal doublet formation rates had normal and nearly normal fasting plasma glucose values. When plasma glucose elevation was more striking no relation between doublet formation and degree of hyperglycaemia was found. Overall correlation between fasting plasma glucose and doublet formation rate is below statistical significance ($r = 0.37$, $t = 1.71$, $p = 0.11$)

creased vibration perception threshold and proteinuria. Non-diabetic subjects (mean age 44 years, range 15–75 years) were known to have normal fasting plasma glucose levels. A smoking history was obtained from all participants; all current tobacco users (pipes, cigars, or cigarettes) were classified as smokers.

Blood collected in EDTA vacuum containers was centrifuged, and the plasma and buffy coat removed. The red cells were then washed twice in high-potassium phosphate-buffered saline (PBS) containing glucose 1 g/l [12]. The cells were then suspended at a concentration of 0.4% in PBS containing bovine plasma albumin 5 g/l and dextran 7.5 g/l (Baker, 100,000–200,000 daltons). A few drops of the red cell suspension were placed on a 25 × 75 mm microscope slide between two 22 × 22 mm number 1 cover slips. A third cover slip was placed over the suspension and the slide then put on the stage of a Leitz trinocular microscope equipped with a GBC Model CTC-3000 video camera whose lens was removed. A 40 × power (high dry) objective was used. Erythrocyte doublet formation (Fig. 1) began after about 15 s and ceased within 4 min as the erythrocytes became adherent to the slide surface. Cells from five serial slide preparations were observed and recorded on half inch (13 mm) VHS tape using a JVC model HR 3600 AU videotape system. A special device imprints the time and number on each frame (Vista Electronics Model 401, La Mesa, California). The five videotape recordings from each subject were examined for doublet formation rate by an observer who had no knowledge of subject classification. Statistical comparisons of the two groups were made using Student's *t* test. Analysis of variance and grouped regression were used to compare the number of doublets formed with severity of diabetic complications. Correlation was tested using the product moment (least squares) technique.

Results

The number of doublets formed by red blood cells from each of the 20 diabetic and 20 non-diabetic subjects studied is shown in Figure 2. The diabetic mean was 4.5 doublets while for non-diabetics the mean was 14.6, a greater-than-threefold difference. Erythrocytes from five diabetics formed no doublets and from another five formed only one or two doublets. The relationship between doublet formation rate and fasting plasma glucose level in the 20 diabetics is shown in Figure 3. A trend to decreased doublet formation with greater elevation of fasting plasma glucose was below statistical significance, but the two highest doublet formation rates were associated with normal and nearly normal fasting plasma glucose levels. Both were Type 2 diabetics (5 and 17 years duration and 96 and 135% of ideal body weight) being treated with insulin. In the first case background diabetic retinopathy had improved markedly following maintenance of normal fasting plasma glucose levels for 6 months. In the second case the majority of fasting glucose values (five of seven) had been less than 8.3 mmol/l for more than 18 months and symptomatic diabetic neuropathy had recently remitted.

The relationship of treatment, type of diabetes and presence of diabetic complications to doublet formation rate is shown in Table 1. No difference was

Table 1. Relationship of specific factors to the number of doublets formed

Factor	Non-diabetic subjects	<i>t</i>	Diabetic subjects	<i>t</i>
Male	11.6 ± 3.5 (5)	1.43	5.6 ± 2.2 (7)	0.7
Female	15.6 ± 1.2 (15)		3.9 ± 1.4 (13)	
Type 1 diabetes			3.4 ± 1.3 (8)	0.8
Type 2 diabetes			5.3 ± 1.8 (12)	
Insulin treatment			5.5 ± 1.6 (14)	1.3
Treatment without insulin			2.2 ± 0.8 (6)	
No detectable complications			3.3 ± 2.5 (7)	0.0
Complications present			5.2 ± 1.3 (13)	
Smokers	18.0 ± 2.2 (7)	2.2 ^a	4.5 ± 2.6 (6)	
Non-smokers	12.8 ± 1.3 (13)		4.5 ± 1.3 (14)	

Results expressed as mean ± SEM; number of individuals shown in parentheses

^a *p* < 0.05

found, nor was there any effect of sex or known duration of diabetes on doublet formation rate (*r* = +0.16, *t* = 0.7). There was no correlation between age and doublet formation rate in either diabetics (*r* = −0.09) or non-diabetics (*r* = +0.03). Smoking had no effect in the diabetic group. The higher doublet formation rate found in the non-diabetics should be treated as equivocal. The probability shown is based on a single *t* test and six tests were made.

Discussion

The marked depression of doublet formation rate observed in diabetes suggests that this technique has potential in studying diabetic erythrocytes. Doublet formation is less influenced by erythrocyte surface-to-volume ratio than techniques that involve cell movement through narrow passages. The force of attraction between erythrocytes was made just sufficient for doublet formation by adjusting the dextran concentration to the lowest possible value. The simplicity of doublet formation rate measurement lends itself both to confirmation of reduced deformability of diabetic erythrocytes and to examination of the specificity of the phenomenon.

Reduction of doublet formation rate in diabetes was found not to be influenced by age, sex, treatment, duration of metabolic disturbance, or the presence of microvascular complications. In contrast to a reported reduction in the filterability of erythrocytes from smokers [13], this study demonstrated a marginally increased doublet formation rate among non-diabetic

smokers, suggesting that the mechanisms involved in the two processes may be different.

The two cells participating in doublet formation change in shape as one cell ascends and slides over the other. Their mutual motion is opposed both by gravity and by the resistance of their cell membranes to bending. Analysis of videotapes suggests that reduced doublet formation in diabetes is due to an increased resistance of the cell membrane to rapid bending rather than to a true membrane rigidity. Most studies reporting reduced erythrocyte deformability in diabetes have exposed the erythrocytes to rapid bending [1–7] while in negative studies membrane bending has been generated more slowly [9, 10]. Increased viscous loss associated with bending has been postulated to explain the limitation of increased viscosity of diabetic erythrocyte suspensions to low shear rates [14].

What is the potential importance of reduced doublet formation rate to diabetes and the development of its late complications? The underlying membrane motion disturbance might not be limited to the erythrocyte; other diabetic cell membranes may also respond abnormally. It is unlikely that the membrane defect alone causes diabetic complications. It is present when no complications are detectable. However, changes in plasma proteins which influence erythrocyte aggregation, even though not specific to diabetes, are known to be associated with earlier evidence of microangiopathy [15]. Erythrocyte aggregation and disaggregation rates diminish when erythrocyte deformability is reduced. Reduced erythrocyte deformability, linked to hyperglycaemia, may combine with non-specific plasma protein changes favouring erythrocyte aggregation to damage blood vessel walls by slowing the red cell disaggregation process.

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