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FLOW BEHAVIOR OF ERYTHROCYTES IN LIVING MICROVESSELS: ANALYSIS OF THE DISTRIBUTION OF DYNAMIC HEMATOCRITS MEASURED *IN VIVO* †

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Abstract—We developed a new method for visualizing the flow behavior of erythrocytes in living microvessels by the fluorescent labeling technique. Intravital microscopic observation of the microcirculation in the rat mesentery using this technique enabled quantitative analyses of a few hydrodynamic aspects of the two-phase blood flow; e.g. diameter–flow relationships in a consecutively branched vascular network and the distribution of erythrocytes in an arteriolar bifurcation.

Key Words: erythrocyte, fluorescent labeling, dynamic hematocrit, microcirculation, intravital microscope, blood flow rate, cell distribution function, preferential flow

INTRODUCTION

Blood flow in the "microcirculation", i.e. the blood circulation in a vascular network on the microscopic scale, provides an interesting subject for analysis from the viewpoint of the two-phase flow. In such a microscopic circulatory system, the blood is not considered as a uniform liquid phase, but the nature of the flow of suspensions of the formed elements of the blood (mostly erythrocytes) in the plasma must be taken into account. The volumetric concentration of erythrocytes in the whole blood, i.e. "hematocrit", is normally about 43% in the systemic circulation, but it has been suggested that the hematocrit in the microcirculation is reduced to as little as about 20%. Thus, the distribution of erythrocytes in the microvasculature, in particular, the hematocrit under flowing conditions in microvessels ("dynamic hematocrit"), becomes an essential hydrodynamic parameter to regulate the blood flow in the living microvessels. In this context, the measurement of the dynamic hematocrit and its analysis are valid contributions to the up-to-date physiology of the microcirculation. However, due to technical difficulties in visualizing the flow of erythrocytes in living tissues under microscopic observation, it is generally hard to measure dynamic hematocrits *in vivo*. To measure this value conveniently in the mesenteric microvasculature of the rat, we tried to use fluorescently labeled erythrocytes as natural tracers of the blood flow. We also analyzed a few quantitative features of the microcirculatory flow using these labeled cells.

MATERIALS AND METHODS

In vivo animal experiments were performed based on the well-established intravital television microscopic observation method of the microcirculation (Ohshima & Sato 1981). A microvascular network of the rat mesentery was observed morphologically under a transilluminated microscope. Also, to visualize erythrocyte movement in the microvessels labeled by a fluorescent dye, the same vascular network was subjected to epiilluminated microscopic observation.

Wistar rats (weighing 250–450 g) were anesthetized with sodium pentobarbital (100 mg/kg). Prior to the microscopic observation, erythrocytes were labeled fluorescently. The blood (1.0 ml) was collected from a catheter placed in a jugular vein into a 3-ml evacuated tube containing heparin

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(100 units) for anticoagulation. The erythrocytes were separated from the plasma by centrifugation and were washed with an isotonic saline solution. The techniques for labeling erythrocytes were practically the same as the method of Tangelder *et al.* (1986) except for a slight modification. These erythrocytes were added to a phosphate-buffered saline solution (PBS), adjusted to pH 7.8, containing 1 mg/ml fluorescein isothiocyanate (FITC). The cell suspensions were incubated at 37°C for 2 h. To remove uncombined fluorescent dyes, the labeled cells were then washed with a saline solution containing 1% bovine serum albumin. The final volume percent of the labeled cells was adjusted to 50% by adding an isotonic saline solution. Complete labeling of erythrocytes by FITC was confirmed under a fluorescent microscope. These suspensions were intravenously injected through a catheter placed in the jugular vein for the following analyses.

The mesenteric microvasculature was observed under an intravital fluorescence microscope system (figure 1). A well-developed vascular network in the mesenteric microcirculation was selected for observation. To measure the diameter of microvessels (D), images of microvessels were recorded on videotapes. The blood pressure was monitored through a catheter placed in the carotid artery during each experiment.

To visualize the fluorescently labeled erythrocytes flowing in the microvessels, the same network was then observed under epiillumination by means of a highly-sensitive SIT-TV camera (Hamamatsu Photonics, C-1000 or C-2400) after the labeled cells were injected (approx. 0.2 ml) through an external jugular vein. The behavior of the labeled cells in the microvascular network was also recorded on videotapes. After these *in vivo* microscopic observations, 2 ml blood were drawn from the catheter placed in the jugular vein to determine hematological parameters *in vitro*, such as the systemic hematocrit and mean corpuscular volume (MCV) of the erythrocytes. Systemic hematocrits were measured using a centrifuge (Kubota, KH-120A) and MCV by an automatic cell counter (Nihon Kohden Co., model MEK-4300). To determine the number of labeled erythrocytes as a fraction (f) of the whole cell population, a droplet of the blood sample was placed on a hemocytometer and the total cell number and the number of labeled cells were counted under transillumination and epiillumination, respectively.

Off-line analyses of the video-recorded images were made to measure the average velocities (v) and the number (n) of erythrocytes passing a luminal cross section. The erythrocyte velocity was calculated by measuring the elapsed time required for a single labeled cell to transverse two luminal cross sections selected arbitrarily, and an average of at least 10 measurements was taken. The dynamic hematocrit (Ht) in each vessel was determined from the following equation, as a

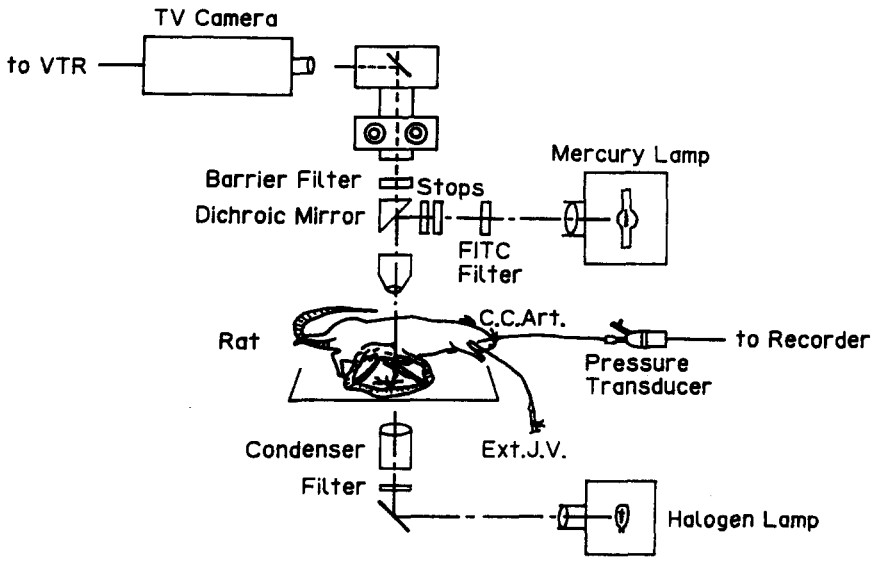

Figure 1. Schematic diagram of the experimental apparatus.

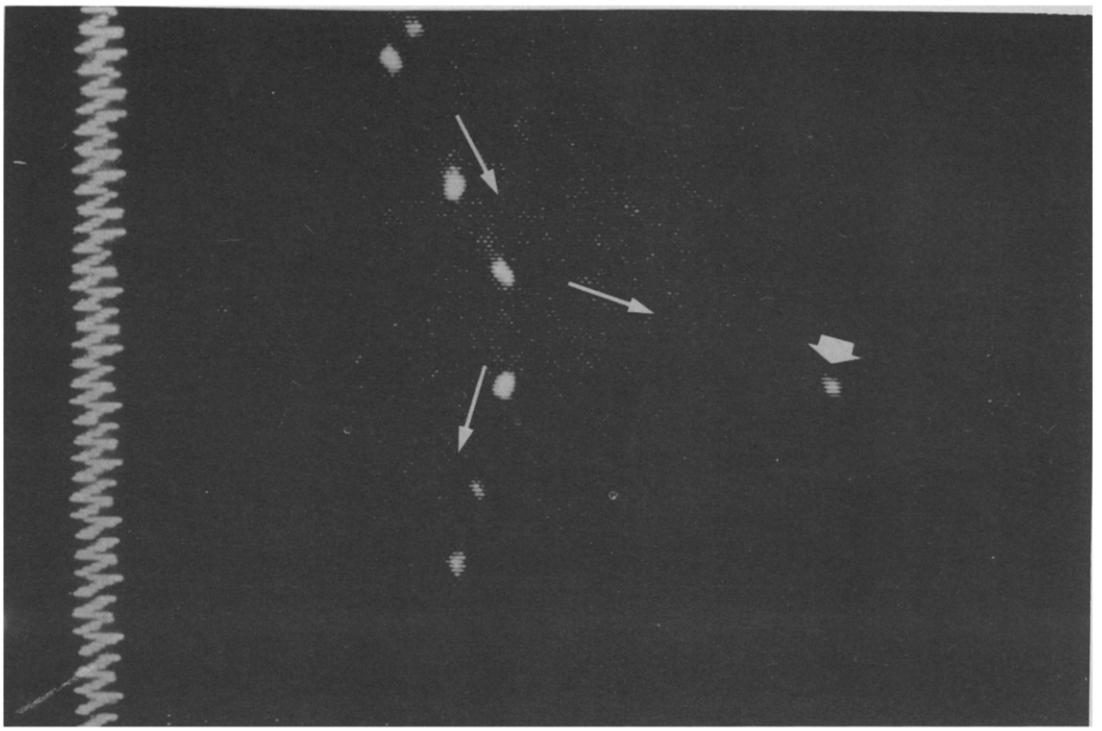

Figure 2. The labeled erythrocytes flowing in the arterioles of the rat mesentery.

ratio of the passage of erythrocytes past a cross section to the average velocity (v) of the whole blood flow:

$$Ht = \frac{\left(\frac{n}{f}\right) \cdot \text{MCV}}{\pi\left(\frac{D}{2}\right)^2 \cdot v}, \qquad [1]$$

where n is the number of labeled cells passing a luminal cross section per unit time (Sarelius & Duling 1982).

RESULTS

The fluorescent labeling of erythrocytes by FITC and the use of the epiilluminated intravital microscope equipped with a highly-sensitive SIT-TV camera enabled us to visualize the flow of erythrocytes in the living microvasculature. These labeled cells were clearly identifiable as moving bright spots, as typically shown in figure 2. In this figure, several labeled cells were observed to flow at almost equal intervals in an arteriole of 15 μm dia. At the bifurcation seen near the center of the figure, a single labeled erythrocyte (shown by a thick arrow) streamed into a small daughter arteriole. Microscopic examination of the blood drawn from the animal after each experiment revealed that the number of labeled cells as a fraction (f) of the whole cell population was 0.05, indicating that the single labeled cell represents 20 erythrocytes nearby. In other words, about 5% of the flowing erythrocytes were labeled fluorescently in the present visualization techniques. Preliminary evaluation of the error in determining the fraction f as the ratio of the number of labeled cells to that of the whole cell population gave an approximate error value of 1.8% ($n = 4$). This value was obtained by directly counting more than 10^6 erythrocytes. Since, in the muscular or parenchymal tissues, other than transparent tissue like the mesentery, identification of the flowing erythrocytes is generally difficult, the labeling technique used in the present study could be applied to the visualization of erythrocyte flow in other less transparent tissues.

The dynamic hematocrit values in several microvascular networks were estimated from [1]. Figure 3 shows a photomicrograph of the rat mesenteric microvasculature observed under the

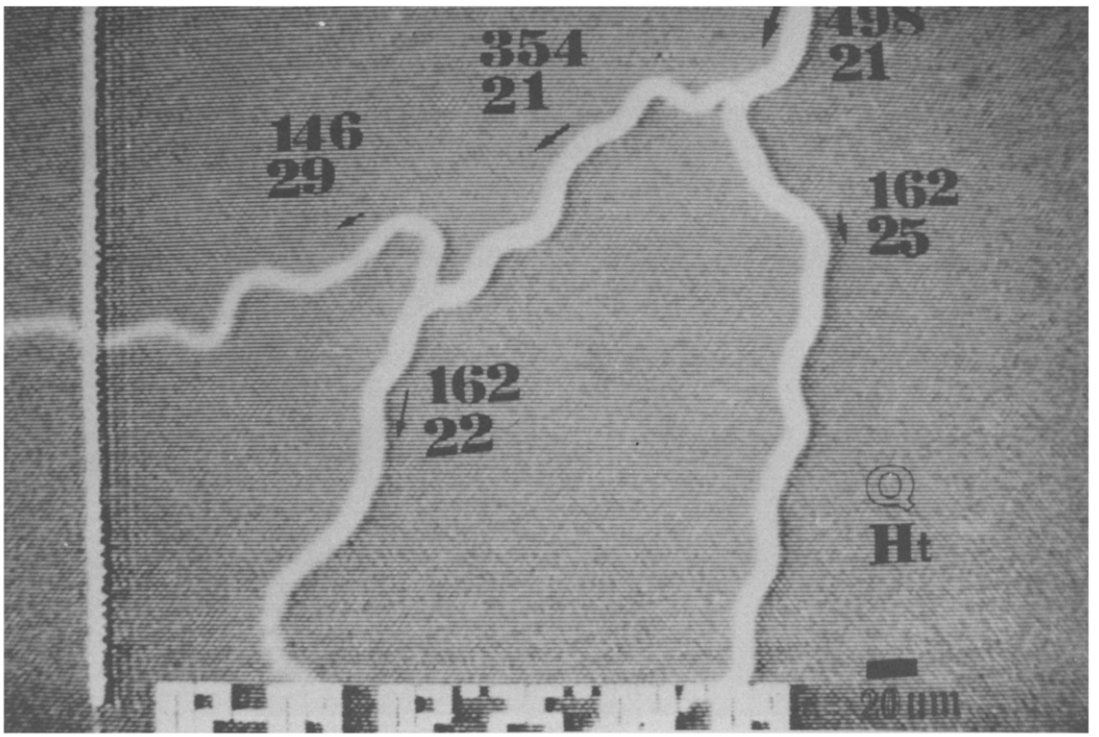

Figure 3. Photograph of the rat mesenteric microvasculature observed under an intravital fluorescence microscope system. The bar indicates 20 μm.

intravital fluorescence microscope system. The upper and lower values indicated in figure 3 represent the blood flow (in units of 10^{-6} mm^3/s) and the calculated dynamic hematocrit (%), respectively, at each portion of the microvessels studied. The number of labeled cells was clearly countable in the diameter range $<30\,\mu$m. The accuracy of the blood flow measurements was assessed by checking a mass balance between measured values of the blood flows determined at 15 bifurcations arbitrarily selected. As compared to the blood flow at the parent (upstream) vessel (100%), a sum of the blood flow at two daughter (downstream) vessels gave an average value of $98.8 \pm 11.1\%$ (mean $\pm$ SD, $n = 15$). Moreover, an experimental error in determining the blood flow at each branch vessel was estimated to be $8.0 \pm 7.9\%$ ($n = 15$). Similarly, a mass balance analysis on the measured fluxes of the labeled cells was performed for the experimentally obtained data at 15 bifurcations. The sum of the flux of the labeled erythrocytes measured at two daughter vessels

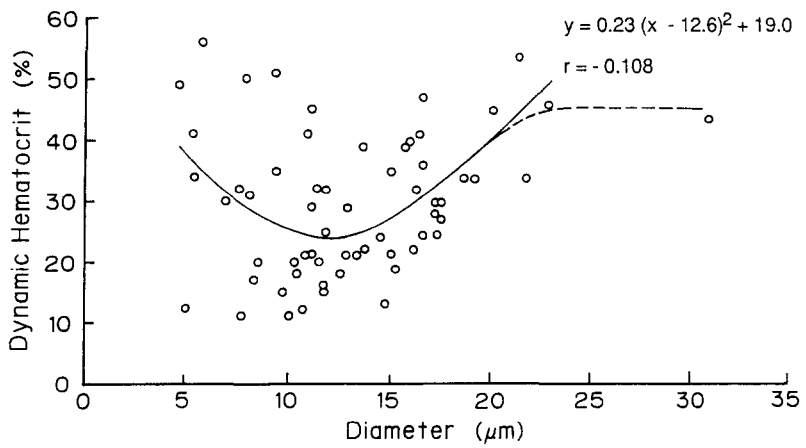

Figure 4. Distribution of dynamic hematocrit as a function of microvessel diameter.

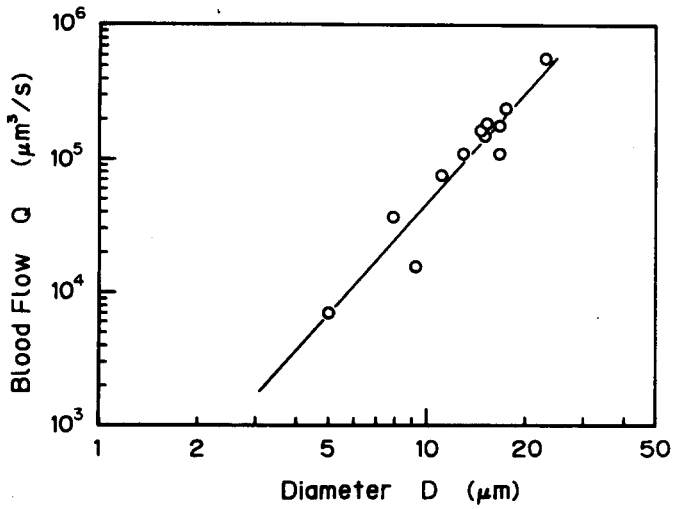

Figure 5. Blood flow as a function of microvessel diameter in a consecutively branching network of the rat mesentery.

resulted in an average of $100.4 \pm 1.6\%$ (mean $\pm$ SD, $n = 15$) as compared to that of the parent vessel (100%). Also, an error in determining the erythrocyte flux at each branch vessel was estimated to be $0.9 \pm 1.3\%$ ($n = 15$). All the results of these error estimations support the soundness of the present method. Figure 4 summarizes the distribution of the dynamic hematocrits as a function of microvessel diameter. The observed values showed considerable scatter, however, an earlier report on the measurement of dynamic hematocrit (Sarelius & Duling 1982) showed a similar degree of scatter in the data; both these results reflect the nature of the dynamic hematocrit. Least-square second-order polynomial curve fitting, as indicated by the solid line in figure 4, revealed that minimum dynamic hematocrit values exist in vessels of approx. 13 μm microvessel diameter.

For consecutively branched microvessels within a particular vascular network, the volumetric blood flow rate (Q) for each vessel was calculated from the measured velocity (v) and the luminal diameter (D). Figure 5 shows an example of such plots in logarithmic coordinates of the blood flow rate against the luminal diameter. The blood flow rate tended to increase with increases in the microvessel diameter over the range 5–20 μm. A least-square fit (the solid line in figure 5) of the data yielded a regression line:

$$\log Q = 2.83 \log D + 1.83 \quad (r = 0.953).$$

The exponent of D for the other four vascular networks ranged from 2.12 to 3.37 (average = 2.69 for all vasculatures); values substantially lower than 4, as expected from the ideal Hagen–Poiseuille law. The exponent obtained in the present study, however, was close to those reported by a couple of papers for the microvascular network (Mayrovitz & Roy 1983; Seki 1993). The deviation of the flow behavior from ideal Hagen–Poiseuille flow appears to be due to the characteristic rheological properties of the blood and the complicated anatomical structure of the vascular network, such as branching and tapering effects.

From measurements of the blood flow rate and the fraction of suspended erythrocytes at the parent and daughter vessels at a vascular bifurcation, we can consider the manner in which the flowing particles are distributed to daughter branches (Chien *et al.* 1984). At an arteriolar bifurcation comprising a parent vessel (P) and two daughter vessels (1 and 2), we can define: two parameters describing the fractional flows of the suspensions and the suspended particles; a fractional erythrocyte flux [$N^* = N_1/(N_1 + N_2)$] and a fractional blood flow [$Q^* = Q_1/(Q_1 + Q_2)$], where N is the erythrocyte flux, Q is the blood flow rate and subscripts 1 and 2 denote daughter

vessels 1 and 2, respectively. Empirically, a relationship between N^* and Q^* is expressed in the form:

$$N^* = \frac{1}{\left(\frac{1-Q^*}{Q^*}\right)^B + 1}. \quad [2]$$

From a plot of N^* and Q^* in logarithmic coordinates, we can determine the empirical coefficient B in [2]. In an extreme case, if the erythrocytes are distributed from the parent to daughter vessels in proportion to the fractional blood flow ratio, the coefficient B should be unity. In contrast, if the greater number of erythrocytes flows into a daughter vessel that has a predominant flow, then $B > 1$. Such a situation is referred to as a "preferential flow". In this case, a plot of N^* and Q^* will give a sigmoidal curve. A plot of measured values of N^* and Q^* obtained experimentally in the present investigation is illustrated in figure 6. At bifurcations which had a daughter vessel in a diameter range $>10\,\mu$m [figure 6(a)], the cell distribution function of N^* and Q^* gave a linear relationship ($B = 1.0$) with an excellent correlation coefficient ($r = 0.97$), whereas at bifurcations with a daughter vessel $<10\,\mu$m [figure 6(b)], the distribution function was also linear ($B = 0.99$), but the data was scattered considerably ($r = 0.88$). These data seemed to suggest that at branches of relatively larger vessels, the erythrocytes flowed into branched vessels in proportion to the flow rate in the branched vessels. However, in smaller branches, some erythrocytes flowed in accordance with a preferential flow, and the rest did not. These diverted results seem to reflect complicated flow behaviors frequently observable in a capillary network: a phenomenon of "plasma skimming" as a consequence of an extreme of preferential flow; and "rouleaux formation", i.e. a concentrated erythrocyte flow stacked face-to-face, as a non-preferential flow in a less perfused capillary.

DISCUSSION

The ultimate purpose of the circulatory system is to supply blood to the peripheral tissues to achieve the transvascular transport of nutrients and metabolic wastes. Therefore, the efficiency of the mass transfer of solute substances such as oxygen, macromolecules etc., is directly affected by the distribution of erythrocytes in microvessels. From these viewpoints, microrheological studies on the behavior of these cells in the microcirculation have been investigated intensively. *In vitro*

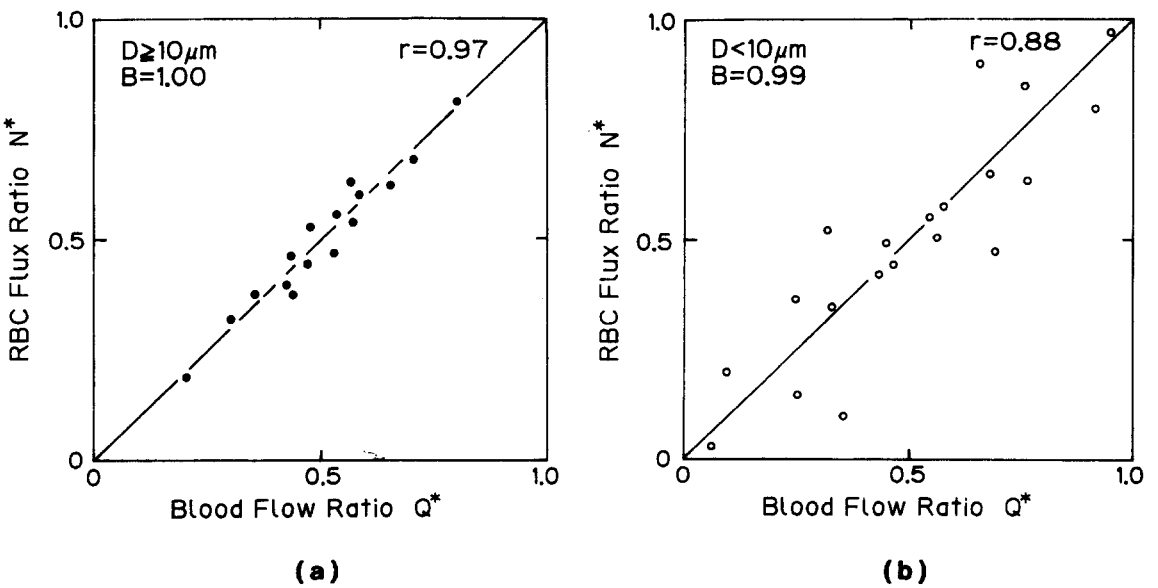

Figure 6. (a) Cell distribution function for vessels $\geq 10\,\mu$m dia. (b) Cell distribution function for vessels $<10\,\mu$m dia.

and *in vivo* experiments revealed some peculiar characteristics of erythrocyte flow in a capillary that have never been observed in the macrocirculation. Fåhraeus (1929) described, for the first time, that both the hematocrit and the apparent viscosity of the flowing blood decreased with decreasing tube diameter. These phenomena are known as the Fåhraeus effect and the Fåhraeus–Lindqvist effect (Fåhraeus 1929), and the detailed mechanisms underlying these effects have also been studied by many investigators (Yen & Fung 1977; Albrecht *et al.* 1979). Previously, we have reported that ratios of capillary hematocrit to discharge hematocrit in narrow glass capillaries showed a characteristic dependence on capillary diameter, showing minimal values at about 13 μm capillary diameter (Ohshima *et al.* 1988). Interestingly enough, this critical value completely agrees with that found in the present investigation, indicating that the microrheological property of the erythrocyte flow observed *in vitro* manifests itself *in vivo* also. In the microcirculatory network, small arterioles (10–20 μm dia) are considered to play a predominant role in the regulation of blood flow, because these arterioles are located immediately before capillaries. The value of 13 μm as a critical diameter seems to imply that beyond this diameter range the flow pattern differs considerably. In fact, close examination of the flow pattern in narrow glass capillaries revealed that the transition from the single-file flow to the multi-file flow occurred at around this diameter range (Ohshima & Sato 1981).

There have been few attempts to measure dynamic hematocrit in small microvessels *in vivo*. The best available method so far reported to measure this property relies on the empirical correlation between dynamic hematocrit and the optical density of the blood; and is based on several assumptions concerning light scattering. It seems difficult to measure the net optical density of erythrocytes in the tissues, especially in the small microvessels, because the light scattering of the surrounding tissue, the vessel wall and the membrane of erythrocytes are not negligible. On the other hand, some investigators have attempted to visualize microvascular blood flow using fluorescently labeled erythrocytes (Tangelder *et al.* 1986; Sarelius & Duling 1982; Sato & Ohshima 1988). Compared with the spectrophotometric method, these visualization methods have the advantage that they allow measurement of a few hemodynamic characteristics other than the dynamic hematocrit. Tangelder *et al.* (1986) have successfully reported the velocity profile of platelets via the fluorescently labeling technique. In the present investigation we also demonstrated the usefulness of the fluorescent labeling technique to obtain pressure–flow relationships, as exemplified by figure 5. Another advantage of the present method in measuring dynamic hematocrits is that multiple measurements of dynamic hematocrit are possible at several portions of the microvessels. The present method is also suitable for analyzing the behavior of the labeled cells at a bifurcation of arterioles or a confluence of venules. The preferential flow analysis attempted in the present study is an example of such applications. Although the exact mechanisms underlying the flow distribution at capillary branches of smaller diameter, as illustrated in figure 6(b), are not clear at present, the results obtained in the present study suggest that the distribution of erythrocytes flowing into capillary branches might be affected by some unknown control mechanisms other than the dimensions or the configurations of the branched vessels.

With respect to the possible change of erythrocyte deformabilities due to fluorescent labeling, we verified that the filtration time of the labeled cells to pass through a 5 μm Nucleopore filter was not significantly different from that of washed erythrocytes. We also confirmed by a confocal laser-scanning fluorescence microscope that the native structure of the labeled cells was not altered by the fluorescent labeling. Close examination of the photomicrography revealed that the labeled cells exhibited a normal biconcave disk shape. In view of these facts, the fluorescent labeling technique used in the present investigation appears to have broad applications in other hemodynamic studies of the microcirculation in addition to that dealt with in this paper.

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