Quantitative Evaluation of Leukocyte Dynamics in Retinal Microcirculation

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Purpose. Leukocyte rheology may play a key role in microcirculation because leukocytes have unique properties, such as large cell volume, high cytoplasmic rigidity, and low deformability. However, only a few methods are available to study the dynamic behavior of leukocytes in retinal microcirculation. The authors developed a new method to analyze directly movements of leukocytes in the retinal vessels of primates.

Methods. Acridine orange, which has been used as a nuclear stain in histochemistry and cytochemistry, was injected intravenously into cynomolgus monkeys for a vital staining of leukocytes. The fundus image was generated with the argon blue laser and a scanning laser ophthalmoscope. The images were recorded on a magnetic tape and evaluated with a personal computer-based image analysis system.

Results. Each leukocyte was recognized as a single fluorescent dot moving in the retinal vessels. It was possible to analyze the spatial and temporal dynamics of individual leukocytes in the capillaries. Some leukocytes passed through the capillaries, plugging transiently under the physiological condition. Leukocytes that stayed in the same position for a few minutes may have stuck to the endothelium as a result of leukocyte–endothelial interactions. In the postcapillary vessels, leukocytes tended to be displaced from the center stream toward the vessel walls. The mean flow velocity of leukocytes in the perifoveal capillary was 0.92 ± 0.32 mm/sec.

Conclusions. This study clearly demonstrated that rheologic behaviors of leukocytes in the retinal microcirculation can be studied through the vital staining with acridine orange in vivo. The authors’ results suggest a potential role of leukocytes in retinal vascular flow disturbances. This study may open the door to the investigation of leukocyte hemodynamics in the retinal microcirculation in vivo. Invest Ophthalmol Vis Sci. 1995;36:123–130.

Various factors, such as blood pressure, vascular network structure, and rheologic properties of blood, determine blood flow in the microcirculation. The contribution of blood rheology to microcirculatory flow disturbances has been discussed extensively. These discussions have focused mainly on erythrocyte dynamics, since the non-Newtonian properties of blood have been mainly attributed to erythrocytes. Recent studies have demonstrated that leukocytes may play a key role in the microcirculation of various organs. Leukocytes are likely to contribute to flow disturbances in the microcirculation under physiological and pathologic conditions, primarily because of their unique properties, including large cell volume, low deformability, and a tendency to adhere to the vascular endothelium. Because the diameter of undeformed leukocytes exceeds that of the average capillary, deformation is necessary to pass through the vessel. However, leukocytes are about 2100 times stiffer than erythrocytes. Capillary plugging by leukocytes has been reported with low perfusion pressure, even under normal conditions. In addition, the adhesive property of leukocytes can cause irreversible capillary occlusion and subsequent vascular endothelial damage.

Little is known about leukocyte rheology in the retinal microcirculation. Recently, Schröder et al demonstrated histologically retinal capillary occlusion...
by leukocytes in the experimental diabetic retinopathy of rats, suggesting the possible role of leukocytes in the pathogenesis of diabetic retinopathy. To our knowledge, no method has been available to study objectively the dynamics of individual leukocytes in retinal vessels in vivo. With the use of the blue field simulation technique, \(^{18-21}\) retinal leukocyte velocity can be estimated under various conditions in human eyes. This method, however, is subjective, and movements of an individual leukocyte can not be studied.

In the present study, we developed a new method of visualizing leukocytes in the retinal vessels and investigated the dynamic behavior of leukocytes in vivo. We used acridine orange, which has been used for nuclear staining in histochemistry and cytochemistry, to stain leukocytes in retinal microcirculation in vivo.

**MATERIALS AND METHODS**

**Animal Preparation**

Three cynomolgus monkeys, weighing 2.5 to 3 kg each, were used. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg) was used for anesthesia. The eyes were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. The ocular surface was then anesthetized with the topical instillation of 0.4% oxybuprocaine hydrochloride, and a contact lens with a radius of curvature of 6.0 mm was placed on the cornea to assure corneal clarity throughout the experiment. The heart rate, tibial blood pressure, and rectal temperature of the monkeys were monitored. Experiments were performed in the animals under the following physiological conditions: heart rate above 60 beats per minute, mean blood pressure above 90 mm Hg, and body temperature between 37°C and 39°C.

**Leukocyte Visualization in the Fundus With Acridine Orange**

Acridine orange has been widely used as a fluorescent probe of nucleic acids in histochemistry, flow cytometry, and cytochemistry. This dye fluoresces by interaction with DNA or RNA, by intercalation, or by electrostatic attractions. When bound to DNA, acridine orange is spectrally similar to fluorescein, with an excitation maximum at 492 nm and an emission maximum at 533 nm. Acridine orange has been used to observe leukocyte rheology in intravital microscopic studies in various organs.\(^{8,11,22}\) This dye also stains nuclei of vascular endothelial cells when injected intravenously.

The fundus images were obtained with a scanning laser ophthalmoscope (Rodenstock Instrument, Munich, Germany). We used the argon blue laser for illumination with the filter setting for regular fluorescein angiography. Acridine orange (WAKO, Osaka, Japan; 0.1% solution in saline) was injected continuously through a saphenous vein at 0.2 ml/min. The maximum dose was 1 mg/kg. We studied the central fundus with a 20° field. Images were recorded on a magnetic tape at a video rate (30 frames/sec).

**Image Analysis Procedures**

The recorded images were digitized through an analog-digital converter board (Video Vision, Radius, CA) and entered into a computer (Apple, Cupertino, CA). The resolution of the digitized image was 640 horizontal and 480 vertical lines (307,200 pixels). The leukocyte flow velocity was measured frame by frame. Briefly, consecutive frames were superimposed using an imaging software (Adobe Photoshop, Adobe Systems). The center position of the leukocyte of the generated image was marked manually on a computer monitor, and the distance of the marked positions was measured in pixels as a straight line. The leukocyte velocity, calculated from the distance of consecutive marked points, was converted into an actual value with the aid of a calibration factor determined in an optically realistic model of the human eye,\(^{23}\) taking into account the size of the primate globe. We selected 50 leukocytes that could be traced from the precapillary arterioles, through the capillaries, and into the post-capillary venules consecutively in the peripheral area. Then, the mean leukocyte capillary velocity was determined by averaging the leukocyte velocities measured with at least 15 consecutive frames.

**In Vitro Leukocyte Staining With Acridine Orange**

To confirm the intravitral staining of leukocytes with acridine orange, the in vitro study was performed as follows. We obtained 2 ml of blood from a monkey and incubated it with 0.5 ml of 0.1% acridine orange solution in saline at 37°C for 30 seconds. The smear specimens of the incubated blood were examined under a fluorescent microscope.

**Retinal Toxicity**

To study a toxic effect of acridine orange on the retina, electroretinograms were recorded 10 minutes before and during and 1 hour after the administration of a 10-fold dose (10 mg/kg) of dye. A week later, regular fluorescein angiography was performed.

**RESULTS**

**Leukocyte Visualization in the Fundus With Acridine Orange**

Each leukocyte was recognized as a single fluorescent dot moving in the retinal vessels. It was possible to
trace consecutively a fluorescent dot moving from the retinal precapillary arteriole to the capillary and to the postcapillary venules in the perifoveal area (Fig. 1). In contrast to sodium fluorescein, acridine orange is not fluorescent unless it binds to DNA inside leukocytes, so retinal vessels were not well visualized at the beginning of the dye injection. Choroidal background fluorescence was negligible, allowing high-contrast pictures of the movements of fluorescent leukocytes.

As the amount of the injected dye increased, the vascular endothelium became fluorescent and gradually obscured the movements of the leukocytes in the capillaries. In contrast, leukocytes were well observed in the larger vessels. When we stopped the dye injection, the staining of vessel endothelium decreased more rapidly than fluorescein angiography.

**Leukocyte Dynamics**

Figure 2 shows experimental records of the flow velocity of three leukocytes passing through the different perifoveal capillaries. The leukocyte flow velocity fell rapidly at the entry of the capillary. Leukocytes flowed typically at a fairly constant speed through the capillaries.

Most of the leukocytes (68%) passed through the capillaries without stopping (Fig. 2A). However, 16 of
FIGURE 2. Leukocyte velocity was measured continuously in the different perifoveal vessels, frame by frame. (A) Typical record of the flow velocity of a leukocyte without plugging. The flow speed falls rapidly at the entry of the capillary and is fairly constant during the passage of the capillary. (B) The leukocyte showed transient capillary plugging. (C) This record shows leukocyte stagnation for 267 msec (eight frames).

FIGURE 3. (A) Most (81%) of leukocytes with plugging stagnate for one frame (33 msec). (B) Leukocytes plugged the capillaries mostly (88%) one or two times from the entry to the exit of the capillary.

50 (32%) leukocytes passed through the capillaries with transient plugging (Figs. 2B, 2C). As shown in Figure 3, the plugging time varied from one (33 msec) to eight frames (257 msec). Most (81%) of the plugged leukocytes stagnated for one frame (33 msec). A few leukocytes stagnated in the same position for more than 1 minute. Leukocytes plugged the capillaries usually (88%) one or two times from the entry to the exit of the capillary.

The mean flow velocity of 50 leukocytes passing through the perifoveal capillaries was 0.92 ± 0.32 mm/sec (mean ± SD). The mean velocities of the leukocytes with plugging and without plugging were 0.68 ± 0.23 mm/sec and 1.11 ± 0.26 mm/sec (mean ± SD), respectively.

In the postcapillary venules, leukocytes were likely to be displaced from the center stream to the vessel walls, and they flowed at a fairly slow speed (Fig. 4). Adhesion was not observed during the experiment.

In Vitro Leukocyte Staining
Fluorescent microscopy showed exclusive staining of the leukocytes in the smear specimen of the monkey.
FIGURE 4. (A) Composite picture of consecutive digital images of a leukocyte during the passage of the postcapillary venule. The venule is shown as a dark tube. A leukocyte flows along the vessel wall in a marginal stream. (arrow) Direction of flow. (B) Close-up image of the same picture. (C) Digital fluorescein angiogram of the same fundus. (arrow) Direction of flow in the postcapillary venule. F = fovea.

blood incubated with acridine orange. Plasma, erythrocytes, and platelets were not fluorescent (Fig. 5).

Retinal Toxicity
The electroretinograms showed no differences in amplitude or implicit time of the a-waves and b-waves taken 10 minutes before and during and 1 hour after a 10-fold dose administration of the dye (Fig. 6). Fluorescein angiography revealed no abnormal findings suggesting vascular damage.

DISCUSSION
The method reported herein demonstrated that it was possible to observe directly and to analyze the rheologic behavior of individual leukocytes in the retinal microcirculation in vivo and has the following advantages: Leukocytes were exclusively fluorescent among the formed elements of blood; acridine orange-filling vessels were not visible; choroidal background fluorescence was negligible; and the method was repeatable after a short interval. These advantages were derived from the unique properties of acridine orange as a nuclear staining and cell-permeable dye. The method allowed the spatial and temporal analysis of individual leukocyte dynamics.

We used scanning laser ophthalmoscope (SLO) titer to visualize fluorescent leukocytes in the fundus. Digital imaging processing using SLO is spatially and temporally limited. The spatial resolution is theoretically about 10 μm because a laser beam is focused at a 10-μm spot on the retina. However, this does not preclude the observance of fluorescent particles smaller than 10 μm. Particles are observed as fluorescent spots similar in size to the laser beam because each time the scanning laser impinges on the leukocytes, it causes them to fluoresce. The recorded SLO images are also temporally limited by video rates (30 frames/sec). The measurement of leukocyte plugging time was restricted by this interval. However, moving particles are more detectable by SLO than by video camera because each spot is scanned with a laser beam for only 100 nsec. Because this is less than 1 μsec, the image of small particles is said to be “frozen.”

Our results showed that the mean capillary leukocyte velocity was 0.92 ± 0.32 mm/sec. With the blue field simulation technique, capillary leukocyte flow velocities of 0.54 to 0.92 mm/sec were reported in normal human subjects. These data are in close agreement with our results despite the different materials used. Considering that the retinal structure of monkeys is similar to that of humans, our procedure may be a promising method to analyze leukocyte rheology in retinal microcirculation.

The origin of hyperfluorescent dots or segments observed with fluorescein angiography using SLO has been controversial. Tanaka et al suggested that they correspond to leukocytes and platelets. We do not agree with their conclusion. We speculate that the hyperfluorescent dots or segments in perifoveal capillaries correspond to cell-free plasma between erythrocytes in the form of rouleaux formation, as other re-
FIGURE 5. Fluorescent microscopy shows exclusive staining of the leukocytes in the smear specimen of the monkey blood incubated with acridine orange. Plasma, erythrocytes, and platelets are not fluorescent.

Searchers have stated.\textsuperscript{26,27} Capillary flow velocities measured by means of the scanning laser technique were reported to be between 2.31\textsuperscript{28} mm/sec and 3.28\textsuperscript{29} mm/sec, higher than the velocities measured in the present study. The discrepancy might be due to the difference between plasma velocity and leukocyte velocity.

Recent findings have indicated that leukocyte plugging in the capillaries is an important rheologic determinant of microvascular blood flow disturbances.\textsuperscript{4,5,7,12} Intravital microscopy has been used to observe directly leukocytes occluding capillaries under low perfusion pressure in skeletal muscle,\textsuperscript{3} brain,\textsuperscript{16} lung,\textsuperscript{15} and kidney.\textsuperscript{6} Other studies\textsuperscript{2,14} have demonstrated that leukocyte plugging is associated with "no reflow phenomenon" during reperfusion after myocardial ischemia. All these studies have shown that every obstructed capillary contained at least one leukocyte, strongly indicating the contribution of leukocytes to capillary occlusion. Some leukocytes (16 of 50) were observed to plug the capillaries transiently under normal conditions, whereas most (68\%) of the leukocytes passed through the capillaries without plugging. To our knowledge, this is the first observation of leukocyte plugging in retinal vessels in vivo. In comparison with the average erythrocyte with a diameter of 8 \mu and a volume of 90 cu \mu,\textsuperscript{50} leukocytes are much larger (180 cu \mu).\textsuperscript{31} Because undeformed leukocyte diameters exceed the average capillary diameter (about 5 \mu), considerable deformation is required for leukocytes to pass through the capillary network. Also, high cytoplasmic rigidity makes it difficult for leukocytes to deform. Leukocyte plugging lasted less than 267 msec. Most (81\%) of the plugged leukocytes stagnated about 33 msec. The leukocytes plugged the capillaries usually (88\%) one or two times from the entry to the exit of the capillary. Normally, the plugging was only occasional and of short duration (0.5 to 5 sec),\textsuperscript{4} probably because a high driving pressure of erythrocytes makes leukocytes deformed and pushes them out through the narrow capillaries at a normal rate of perfusion. Under reduced perfusion pressure, the leukocytes are insufficiently deformed and become trapped, which may increase the chance of leukocyte–endothelium interactions. In our experiment, leukocytes rarely stagnated for more than 1 minute. The SD of the mean leukocyte capillary velocity in the perifoveal area (0.92 ± 0.32 mm/sec, mean ± SD) was 35\%.

Before

During

After

![Graph](image-url)

FIGURE 6. Electretinograms show no differences in amplitude or implicit time of the a-waves and b-waves taken 10 minutes before and during and 1 hour after a 10-fold dose administration of the dye.
Leukocyte Dynamics in Retinal Microcirculation

Leukocytes because the mean capillary flow velocity of the plugged leukocyte (0.68 ± 0.25 mm/sec, mean ± SD) was about half that of unplugged leukocytes (1.11 ± 0.26 mm/sec, mean ± SD). The mean capillary flow velocities of unplugged leukocytes varied from 0.69 to 1.74 mm/sec. Other explanations for the considerably large SD may be attributed to differences in capillary perfusion pressure; differences in the retinal microvasculature, such as diameter; differences in leukocyte size (monocytes are the largest and lymphocytes are the smallest); preferential channels for leukocytes (leukocytes tend to be shunted through channels with the fastest flow).8

In postcapillary vessels, leukocytes moved fairly slowly along the vessel walls in a marginal stream and did not adhere to the endothelium. The findings can be explained by the fact that faster erythrocyte flow pushes leukocytes toward the walls. The chance of leukocyte–endothelial interactions will increase. Recent evidence has indicated that the postcapillary venule is the preferential site of the leukocyte adhesion.9,10,12 Shear forces may be another factor because the postcapillary venule is the site of the first major decrease in vessel wall shear stress.

Acridine orange has long been used to stain and visualize leukocytes by intravital microscopy.3,11,22 The dye is suggested to be an antibacterial, mutagenic, carcinogenic, or antimutagen agent.32 The phototoxic effect also has been observed after the exposure of acridine orange-stained cells to light. We think it is difficult to apply acridine orange to humans. However, it has been reported that these cytotoxic effects may be due to nonpurified preparations of acridine orange.32 In the present study, no considerable toxic effects were observed on leukocyte movements, retinal function, or retinal microcirculation, as evaluated by ERG a-waves and b-waves and fluorescein fundus angiography.

In conclusion, the present method provided detailed information about individual leukocyte behavior in the retinal microcirculation. Additionally, our results suggested a possible role of leukocytes in blood flow disturbances of the retina. The method provides a new way to investigate leukocyte rheology in the retinal microcirculation in vivo.

Key Words

leukocyte rheology, acridine orange, blood flow, scanning laser ophthalmoscope, capillary plugging

References

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