Evaluation of Leukocyte Dynamics in Choroidal Circulation with Indocyanine Green–Stained Leukocytes

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PURPOSE. To develop a new method with which to visualize leukocytes moving through the choroidal vessels of pigmented animals and enable the evaluation of leukocyte dynamics in the choroidal microcirculation.

METHODS. Pigmented rabbits and monkeys were used in this study. Leukocytes, collected by centrifugal separation of autologous blood, were stained with indocyanine green (ICG) dye. The ICG-stained leukocyte fluid was injected into the vein, and the fundus image was obtained with a scanning laser ophthalmoscope. The image was recorded on videotapes and analyzed with a personal computer-based image analysis system.

RESULTS. In pigmented rabbits, fluorescent leukocytes moving in the choroidal circulation were clearly visible for more than 1 hour. In monkeys, distinct fluorescent dots were seen moving approximately 50 to 200 μm in the foveal avascular zone for more than 30 minutes after the injection of the ICG-stained leukocyte fluid. Dim fluorescent dots were seen moving in the fundus. Although the movement of these dim dots was difficult to trace, they seemed to be moving in the choroidal vessels. In the rabbits, the mean flow velocity of leukocytes moving without plugging was $0.48 \pm 0.14$ mm/sec in the peripheral choriocapillaris. In the monkeys, the mean flow velocity of distinct fluorescent leukocytes without plugging was $2.45 \pm 0.48$ mm/sec in the posterior choroid.

CONCLUSIONS. In pigmented rabbits and monkeys, this method allows visualization of leukocytes passing through the choroidal vessels and provides a new way to investigate, noninvasively and in vivo, leukocyte dynamics in the choroidal microcirculation. (Invest Ophthalmol Vis Sci. 2000;41: 2844–2848)

Leukocytes in the retinal and choroidal circulation have been investigated in vivo, because leukocytes may play a key role in the microcirculation.1–4 Acridine orange digital fluorography enables evaluation of leukocyte dynamics in the retinal circulation under physiological and pathologic conditions.1 Because acridine orange is spectrally similar to fluorescein and stains all nuclear material including the retinal pigment cell nuclei, leukocytes circulating in the choroid cannot be seen. The use of this method is limited to animal studies, because acridine orange is carcinogenic and may be phototoxic to cell lysosomes. Matsuda et al.2 reported that leukocytes, stained with indocyanine green (ICG) dye, were observed as hyperfluorescent dots in the choroidal microcirculation of nonpigmented rats by using regular ICG angiography with a scanning laser ophthalmoscope (SLO). However, it is well known that regular ICG angiography does not enable the visualization of leukocytes in the choroid of other animals, such as rabbits and monkeys, or in humans. In the report by Matsuda et al., visualization of leukocytes in the choroidal circulation of pigmented rats was not possible because of significant pigment in the retinal pigment epithelium (RPE) and choroid. Yang et al.3,4 developed a new method, called fluorescein leukocyte angiography and studied the leukocyte dynamics in the retinal and choroidal circulation. However, it is also impossible to investigate the choroidal circulation of pigmented animals with this method, because of staining with fluorescein sodium and the pigmentation of the RPE and choroid. To our knowledge, no method has yet been available to study in vivo leukocyte dynamics in the choroid of pigmented animals, such as pigmented rabbits and monkeys, or in humans.

We developed a new method with which to directly visualize leukocytes in the choroid of pigmented animals and investigated leukocyte dynamics in the choroidal circulation of pigmented rabbits and monkeys through autologous leukocytes stained with ICG dye. Our method, called indocyanine green leukocyte angiography (ILA), can be applied to the evaluation of leukocyte dynamics in the choroidal circulation and may be feasible for use in humans.

MATERIALS AND METHODS

Animal Preparation

Two pigmented rabbits (Japan Black) with body weights of approximately 3 kg and two adult monkeys (Macaca fuscata) with body weights of 6.0 kg and 6.4 kg were used in this study. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-five mg/kg pentobarbital sodium (Nembutal;
Abbott Laboratories, North Chicago, IL) was used for anesthesia and was supplemented as needed through a vein during the experiment.

**Indocyanine Green Leukocyte Angiography**

Ten to 20 ml blood was withdrawn from a vein (in rabbits from the ear vein; in monkeys from the cubital vein) into a sterile test tube. The blood was mixed with a mixture of Ficoll and metrizoate (Mono-Poly Resolving Medium; Dainippon Pharmaceutical, Osaka, Japan) and separated using a centrifuge at 1800 rpm for 30 minutes. Most of the plasma and the bulk of erythrocytes were removed and the white-coat layer of leukocytes was mixed with 0.01 ml (in rabbits) or 0.05 ml (in monkeys) ICG solution (Diagnostreen injection; Dainich Pharmaceutical, Tokyo, Japan). Additional centrifugal separation of the leukocyte fluid at 1500 rpm for 5 minutes was performed to collect ICG-stained leukocytes more densely. Three milliliters of phosphate-buffered saline (PBS) was added to the ICG-stained leukocytes. The whole leukocyte fluid was estimated to contain 10 to 60 million leukocytes by counting the leukocytes in 0.1 μl of the leukocyte fluid.

Pups were diluted using 0.5% tropicamide ophthalmic solution. The leukocyte fluid was injected into the ear vein (in rabbits) or the antecubital vein (in monkeys), and the fundus images were obtained with infrared laser and an SLO (model 101; Rodenstock Instrument, Munich, Germany). The SLO was operated using a 40° field size in rabbits or a 20° field size in monkeys. The images, obtained at 30 frames/sec, were recorded on SVHS videotapes. Twenty minutes after the injection, simultaneous fluorescein and ICG angiography, showing retinal vascular landmarks, was performed to locate ICG-stained leukocytes in the monkeys.

**Image Analysis**

Images were analyzed with a computer-based image analysis system. Consecutive images were captured with an analog–digital converter board (Dig98; Ditect, Tokyo, Japan) loaded into a personal computer (PC9821Xa; NEC, Tokyo, Japan). Consecutive images were superimposed using original software running on Windows 95 (Microsoft Japan, Tokyo, Japan) so that the movement of fluorescent dots could be traced on one image. The center portion of leukocytes was marked manually on a computer monitor, and the distance between consecutively marked positions was measured in pixels as a spline curve, which has been applied to tracer flow extraction in arterial blood flow studies. The flow velocity, calculated in pixels from the distance of consecutive portions, was converted into a real value with the aid of a calibration factor determined in an optical realistic model of the human eye for the monkey experiment. For the rabbit, a real size was measured in vivo after insertion of a thin ruler on the surface of the retina using the SLO. The velocity of 10 leukocytes moving in the choroidal vessels was measured in each animal.

**RESULTS**

A few seconds after the injection of the leukocyte fluid, relatively weak ICG fluorescence of plasma was first visible. For the initial 3 minutes in rabbits (5 minutes in monkeys), the transit of phase similar to regular ICG angiography was seen, and the choroidal arteries and veins were identified by their diffusion time. After that, ICG fluorescence of plasma significantly decreased, and choroidal arteries and veins in rabbits (retinal and choroidal in monkeys) were outlined as negative features against the background.

**Pigmented Rabbits**

Composite photographs of early-phase images and late-phase images were used for the differentiation between arteries and veins. Many fluorescent dots became distinctly visible approximately 5 minutes after injection. For more than 1 hour, each ICG-stained leukocyte passing through the choroidal circulation was distinctly observed as a single fluorescent dot moving in the choroidal vessels. Because choroidal arteries and veins were outlined as negative figures against the background, it was possible to observe these leukocytes moving rapidly in the choroidal arteries, passing very slowly through the choroidal capillaries and draining into the choroidal veins at increasing velocity (Fig. 1). Transient plugging, defined as absence of flow over two intervals between frames, was frequently seen during passage of leukocytes through the capillaries. Most of the plugged leukocytes stagnated for less than 1 second.

The flow velocities of the fluorescent leukocytes that did not plug decreased rapidly at the entry point of the choroidal capillaries and were constant during passage through the capillaries. The flow velocities of leukocytes that did not plug, which passed through the peripheral capillaries, ranged from 0.26 to 0.93 mm/sec (mean velocity ± SD, 0.48 ± 0.14 mm/sec).

**Monkeys**

Approximately 10 minutes after the injection, fluorescent dots became evident in the posterior region, and afterward each leukocyte was distinctly recognized as a single fluorescent dot in the posterior region for more than 30 minutes by using a 20° field size. These fluorescent leukocytes were located with retinal vascular landmarks and negative images of the choroidal vessels.

In the monkey, there were two patterns of leukocytic movement. In one pattern, fluorescent dots moved from the retinal artery toward the perifoveal network, passed through the perifoveal capillaries, and drained into the retinal veins (Fig. 2). It was possible to consecutively trace the movement of the fluorescent dots moving through the retinal circulation. The mean flow velocity of leukocytes passing through the perifoveal retinal capillaries was 1.10 ± 0.37 mm/sec. In the other pattern, fluorescent dots appeared abruptly on a video monitor, moved approximately 50 to 200 μm, and disappeared abruptly (Fig. 3). Because the latter pattern was often observed in the foveal avascular zone (Fig. 4), the fluorescent dots moving in this pattern represent ICG-stained leukocytes in the choroidal circulation. The mean flow velocity passing the choroidal vessels was 2.45 ± 0.48 mm/sec. This was calculated based on two or three consecutive images by which the movement of each dot could be traced. In both patterns, transient plugging was sometimes observed. Additionally, dim fluorescent dots were observed moving too fast to be recognized in the still image. Although the movement of these dim dots was difficult to trace, it was substantially different from that of
bright dots moving in the retinal circulation. These dots seemed to be moving in the choroidal vessels.

**DISCUSSION**

In regular ICG angiography, fluorescence of plasma masks leukocyte fluorescence in the early to middle phase of the angiography, and in the late phase, in which the fluorescence of plasma disappears, leukocytes with decreased fluorescence cannot be recognized because of blockade by much pigment of the RPE and choroid. ILA is based on the differential fluorescent staining among blood contents, in that leukocytes are intensely stained with ICG dye. In the middle- to late-phase of ILA (in rabbits, 5 minutes to more than 1 hour after the injection of ICG-stained leukocyte fluid; in monkeys, 10–40 minutes after that), leukocytes can be recognized as hyperfluorescent dots in the choroid. ILA can be also applied to nonpigmented animals. In our preliminary study, clear visualization of leukocytes was confirmed in the choroidal circulation of albino rabbits.

The flow velocity of leukocytes moving in the choroidal vessels was measured in the present study. The fastest velocity that could be measured by using the viewing system in the present study was estimated to be 30 mm/sec. In rabbits, fluorescent dots in the choroidal arteries were clearly observed. However, the movement in the arteries was too fast to be traced on a monitor, and it was difficult to evaluate the velocity of them accurately. Koyama et al. reported that the flow velocity of erythrocytes moving in the choriocapillaris of albino rabbits, which was measured by using high-speed videography taken through a scleral window, ranged from 0.28 to 2.11 mm/sec and that leukocytes moved more slowly than erythrocytes. Matsuda et al. reported that leukocyte velocity in the choroidal capillaries was $0.74 \pm 0.06$ mm/sec in the nonpigmented rats. In the present study, the mean velocity was $0.48 \pm 0.14$ mm/sec. The discrepancy may be due to the difference in species or measurement sites.

In primates, little is known about leukocyte rheology in the choroidal circulation, and the flow velocity of leukocytes in the choroid is unknown, because there is no available method. In contrast, the mean flow velocity of leukocytes passing through the retinal perifoveal capillaries was reported to be $0.92 \pm 0.32$ mm/sec in monkeys and $1.41 \pm 0.29$ or $0.54 \pm 0.19$ mm/sec in humans. These data are in close agreement with our data ($1.10 \pm 0.37$ mm/sec), despite the different methods used. In contrast, the mean velocity of leukocytes moving in the posterior choroid of the monkeys was $2.45 \pm$
0.48 mm/sec in the present study, approximately two times higher than the velocity in the retinal capillaries. The large choroidal vessels are at a depth different from that of the choriocapillaris. We observed leukocyte flow at a constant focal plane during a sequence of data collection. Thus, we may be biasing our observations to leukocytes that do not deviate significantly from the plane.

In the monkeys, the fluorescent leukocyte appeared abruptly on a video monitor, moved approximately 50 to 200 μm corresponding to the dimension of the lobules of the choriocapillaris,9 and disappeared abruptly. These movements seem to reflect the choroidal vasculature, in that feeding arteries and drainage veins are at a right angle to the choriocapillaris. Leukocytes in the choroidal arteries and veins move much faster than those in the capillaries. Indeed, we observed dim fluorescent dots moving too fast to be recognized in the still image, the quality of which was much lower than the real-time image. Fluorescence of leukocytes in the artery or vein of the deeper choroid may be more strongly blocked by much pigment of the choroid than that in the capillaries. Thus, we supposed that distinct fluorescent dots represent leukocytes passing through the choroidal capillaries. Japanese monkeys (Macaca fuscata) used in this study have much pigment in the choroid compared with humans. Chang et al.10 described that they used a higher dose of ICG (2 mg/kg) in the rhesus monkeys (Macaca mulatta) to gain high-contrast ICG angiographic images, compared with a dose of ICG (1 mg/kg) in human eyes. More distinct images of fluorescent leukocytes in the choroidal circulation might be gained in human eyes or other monkeys with less pigment. Further studies are necessary to prove the assumption that the distinct fluorescent spots seen in the present study are leukocytes passing through the choroidal capillary.

Transient plugging can be seen in the capillary under physiological and pathologic conditions.1–4 As described, transiently plugged leukocytes in the choriocapillaris were frequently seen in rabbits. The majority of them remained stagnant for less than 1 second. Plugging was sometimes seen in the monkeys. However, it is sometimes difficult to distinguish whether true plugging occurs or whether it is artifactual, due to movement of the leukocyte perpendicular to the choriocapillaris.

To our knowledge, this study is the first to visualize leukocytes moving in the choroidal microcirculation of pigmented animals including monkeys. ILA is available to evaluate leukocyte dynamics in vivo and noninvasively and would be useful for the experimental study of choroidal hemodynamics in pigmented subjects under pathologic conditions. Additionally, this method may be applicable in humans because of the minimal toxicity of ICG and holds a possibility of disclosing a role of leukocytes in the pathogenesis of various ocular diseases.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)

**Figure 3.** Composite photograph of three consecutive images (with an interval of 1/30 second) demonstrating a fluorescent dot moving in the choroidal circulation. *Arrow:* Direction of the movement of the dot. The dot appeared abruptly in the video monitor, moved 143 μm in 1/15 second, and disappeared abruptly. Its flow velocity was 2.15 mm/sec. Bar, 0.2 mm.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)

**Figure 4.** Simultaneously acquired ICG leukocyte and fluorescein angiograms in the macular region of a monkey eye. (A) ILA showing fluorescent ICG-stained leukocytes. (B) Fluorescein angiogram (FA) taken simultaneously with ILA. (C) Combined image showing the fluorescence of the ILA with green and that of the FA with red. Three ICG-stained leukocytes (*arrows*) are located within the foveal avascular zone. Bars, 0.2 mm.
References