In Vivo Three-Dimensional Evaluation of Leukocyte Behavior in Retinal Microcirculation of Mice

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Purpose. To evaluate new physiologic and three-dimensional methods for monitoring leukocyte behavior in mouse retina.

Methods. Endotoxin-induced uveitis (EIU) was produced in mice by footpad injection of lipopolysaccharide (LPS). Leukocytes were labeled with acridine orange (AO). Leukocyte rolling in the retinal microcirculation was evaluated in vivo with AO digital fluorography. The number of migrated leukocytes was counted in flatmounted retina. The behavior of leukocyte migration was observed three-dimensionally at the time of peak migration. After leukocytes were labeled with AO, the mice were perfused with rhodamine-labeled concanavalin A lectin to stain the vascular endothelium. Leukocyte migration into the retina was then monitored three-dimensionally with confocal microscopy, and the velocity of the migration was measured.

Results. Both leukocyte rolling and migration peaked at 48 hours after LPS injection. Leukocytes were seen to extravasate from the deeper capillary layers and to migrate toward the outer layer of the retina. The traveling velocity of extravasated leukocytes in retinal tissue was 2.0 ± 0.1 μm/h.

Conclusions. New methods have been demonstrated for the three-dimensional and quantitative evaluation of leukocyte behavior in mouse retina. (*Invest Ophthalmol Vis Sci.* 2004;45: 4197–4201) DOI:10.1167/iovs.04-0192

Leukocytes play an important role in the pathogenesis of inflammation.[1] Upregulation of leukocyte–endothelial cell interactions in the retina is associated with various inflammatory conditions.[2–7] At the site of inflammation, leukocytes in the microcirculation interact with adhesion molecules of endothelial cells, which leads to rolling, adhesion, and migration of the leukocytes.[1] These leukocytes subsequently release cytokines and produce proteases and superoxide radical species, which also participate in the cascade of inflammation.[8,9] The reactions of these leukocytes cause the inflammatory tissue injury or endothelial cell injury that ultimately leads to elevated vasopermeability.[6,10,11] Consequently, it is important to evaluate the behavior of leukocytes in vivo.

In vivo methods to evaluate leukocyte–endothelial cell interactions quantitatively were established in rat retina.[3–5,7,12] Using these methods, we can physiologically evaluate the number of rolling leukocytes and the number of migrated leukocytes in rat retina. However, we have been unable to evaluate leukocyte–endothelial cell reactions in mouse because of the difficulty in maintaining transparency of mouse optic media. With recent progress in gene technology, represented by various knock-out or transgenic mice, expression of the target genes can now be easily modified in mice.[13–15] Accordingly, with in vivo observations of leukocyte rolling and migration in mouse retina, we can accurately evaluate the genetic regulation of leukocyte behavior in inflammatory responses.

Several other methods are available to evaluate the behavior of migrated leukocytes.[16–20] However, to our knowledge, no method is able to observe three-dimensionally the behavior of leukocytes extravasated into retinal tissue in vivo. In the mouse, major vessels of the retina exist in the nerve fiber layer, and three capillary layers are present between the nerve fiber layer and the inner nuclear layer.[21] The three-dimensional observation will reveal from where the leukocytes migrating in the retina are derived, for which direction they are bound for, and how fast they migrate into the retinal tissue. Investigation of the distribution of migrated leukocytes may deepen our understanding of the pathogenesis of the retinal damage caused by these leukocytes. In addition, the evaluation of leukocyte activity is aided by measurements of the behavior of migrated leukocytes in the retina.

This study reports on new quantitative in vivo methods for evaluating leukocyte migration in mouse retina.

Methods

Animal Model

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male pigmented C57BL/6J Jms Slc mice (8–10 weeks old; $n = 114$) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Endotoxin-induced uveitis (EIU) was produced in mice by injecting 100 μg of lipopolysaccharide (LPS; *Salmonella typhimurium*; Sigma Chemical, St. Louis, MO) diluted in 0.1 mL sterile saline into one hind footpad of each animal. Control animals received a footpad injection of saline alone. All mice were maintained in an air-conditioned room with a 12-hour light/12-hour dark cycle and given free access to water and food until they were used for the experiments.

Leukocyte Rolling in Mouse Retina

To evaluate leukocyte rolling during an episode of EIU in mouse retina, we used acridine orange digital fluorography, a method that has been used to evaluate leukocyte–endothelial cell interactions in rat retina, with modifications.[3–5,7,12,22,23] In brief, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany), coupled with a computer-assisted image analysis system (Radius, San Jose, CA), made continuous high-resolution images of the fundus stained by metachromatic fluorochrome acridine orange (AO; Wako Pure Chemicals, Osaka, Japan), which emits a green fluorescence when it interacts with DNA. An argon blue laser was used as the illumination source, with a regular emission filter for fluorescein angiography,

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because the spectral properties of leukocytes stained with AO are similar to those of sodium fluorescein. For further analysis, the obtained images were recorded on an S-VHS videotape at the rate of 30 frames/s.

AO digital fluorography was performed at 4, 12, 24, 48, 72, and 96 hours after LPS injection. Six different mice were used at each time point.

Immediately before AO digital fluorography, the mice were anesthetized with xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), and their pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. Body temperature was maintained between 37°C and 39°C throughout the experiment. A contact lens was used to retain corneal clarity throughout the experiment. Each mouse had a catheter inserted into the femoral vein and was placed on a movable platform. AO (0.01% solution in saline) was injected continuously through the catheter for 1 minute at a rate of 1 mL/min. Rolling leukocytes were defined as leukocytes that moved at a velocity slower than that of free-flowing leukocytes. The number of rolling leukocytes was calculated from the number of cells per minute crossing a fixed area of the vessel at a distance two disc diameters from the optic disc center. The flux of rolling leukocytes for each mouse was defined as the average of individual numbers of rolling leukocytes seen in all major veins.

The diameters of major retinal vessels were measured at two disc diameters from the center of the optic disc in monochromatic images recorded before AO injection. Each vessel diameter was calculated in pixels as the distance between the half-height points determined separately on each side of the density profile of the vessel image and converted into real values using the calibration factor. The averages of the individual arterial and venous diameters were used as the arterial and venous diameters for each mouse.

Number of Leukocytes Migrating in Mouse Retina

The number of leukocytes migrating in mouse retina was evaluated in flatmounted retina after AO digital fluorography. Six different mice were used at each time point. Thirty minutes after the injection of AO, one eye from each of six mice was enucleated. Blood was collected to count the number of leukocytes in the peripheral blood with a hematology analyzer (ERMA, Tokyo, Japan). After the experiment, each rat was killed with an overdose of anesthesia.

The retina was carefully removed, and flatmounts were prepared using a fluorescence anti-fading medium (Vector Laboratories, Burlingame, CA). The retinas were then observed using fluorescence microscopy (FITC filter; Olympus Optical, Tokyo, Japan), and the numbers of fluorescent dots in the retina within four separate circles of 800 μm diameters next to the optic disc were counted. The average of the numbers within the four circles was considered the number of leukocytes migrated in the retina for each mouse.

Three-Dimensional Observation of Leukocyte Migration

The experimental mice were anesthetized 48 hours after LPS injection; control animals were anesthetized 48 hours after footpad injection of saline alone. AO was injected continuously through the catheter for 1 minute at a rate of 1 mL/min.

The animals were perfused with rhodamine-labeled concanavalin A lectin (ConA; Vector Laboratories), 10 μg/mL in phosphate buffered saline ([PBS], pH 7.4) at 30 minutes, 2 hours, 6 hours, and 10 hours after AO injection. ConA was used to label vascular endothelial cells. Under deep anesthesia, the chest cavity was opened and a 24-gauge perfusion needle was introduced into the aorta. Drainage was achieved by opening the right atrium. The animals were then perfused with 10 mL PBS to wash out blood cells in the vessels. After PBS perfusion, the animals were perfused with 10 mL rhodamine-labeled ConA; residual unbound Con A was removed with 2% paraformaldehyde perfusion. Immediately after perfusion, the retinas were carefully removed and flatmounts prepared using a fluorescence anti-fading medium (Vector Laboratories). The retinas were surrounded by tape to shield the tissue from direct pressure by the cover glass.

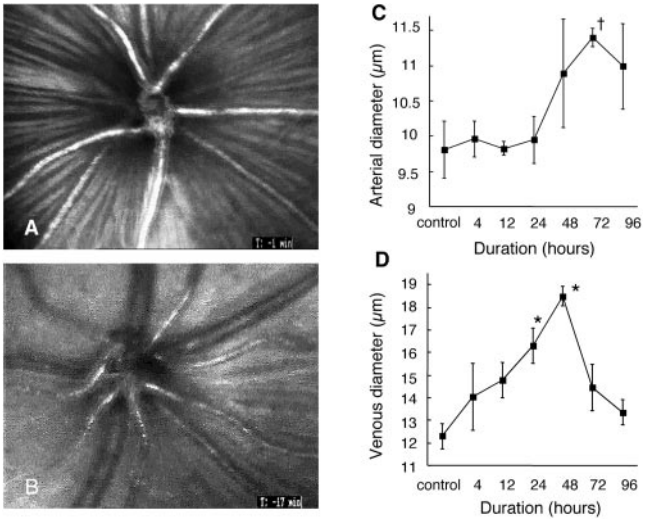

FIGURE 1. Digitized monochromatic images of major retinal vessels obtained with a scanning laser ophthalmoscope in a control mouse (**A**) and a mouse at 48 hours after LPS injection (**B**). The major vessels showed significant vasodilation at 48 hours after LPS injection. Time course of major retinal arterial (**C**) and venous (**D**) diameters after LPS injection. Values are mean ± SEM, $^{*}P < 0.01$, $^{\dagger}P < 0.05$, compared with control mice.

The retinas were then observed with a confocal microscope (LSM5Pascal; Carl Zeiss, Jena, Germany) with both FITC filter (excitation, 488 nm; detection, 505–530 nm) and rhodamine filter (excitation, 543 nm; detection, >560 nm), coupled with a computer-assisted image analysis system. The slice images of the retina, 460.6 μm^2 with magnification of 200, was obtained at 2.25 μm intervals from 100 to 160 μm in thickness from the surface of the retina. Using the analyzing software, we could obtain a reconstructed three-dimensional view of the retina. The migrated leukocytes were expressed as green fluorescent dots, and the vascular network was defined as a red fluorescent structure.

Velocity of Leukocyte Migration

The distance of the migrated leukocyte from the third (and most outer) capillary plexus was measured perpendicular to the retinal surface; this was considered to be the extent of leukocyte migration. One leukocyte from each of six mice at each time point was investigated. The distance the leukocyte had migrated by each time point was defined as the average of individual lengths of leukocyte migration. To determine the velocity of leukocyte migration, the extent of leukocyte migration 10 hours after AO injection was divided by the time (μm/h).

Statistical Analysis

All values were expressed as mean ± SEM. Data were analyzed using the unpaired *t*-test. Differences were considered statistically significant when the probability values were <0.05.

RESULTS

Physiologic Data and Vessel Diameters

Figures 1A and 1B show characteristic fundus images of a control mouse and a treated mouse, respectively, 48 hours after LPS injection. Figures 1C and 1D indicate changes of major retinal vessel diameters in mice at various time points after LPS injection. In arteries (Fig. 1C), significant vasodilation occurred and peaked at 72 hours after LPS injection (116.2%,

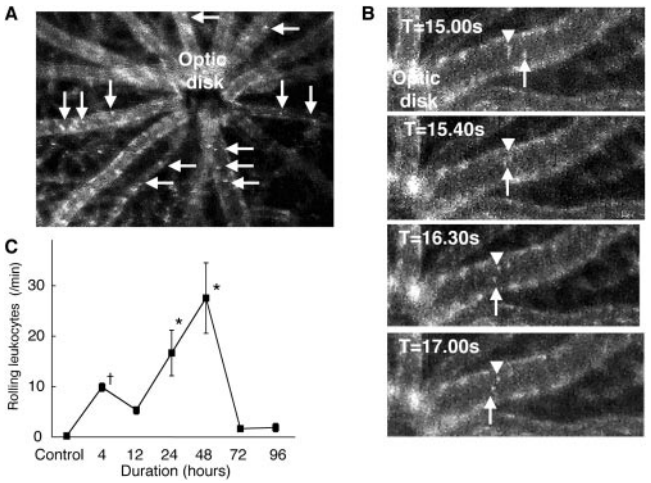

FIGURE 2. **(A)** Leukocytes were stained selectively among circulating blood cells. Nuclei of vascular endothelial cells also were stained. Among free-flowing leukocytes, many rolling leukocytes were observed along the major retinal veins (*white arrows*). No rolling leukocytes were seen along the major retinal arteries. **(B)** Two leukocytes (*white arrow* and *white arrowhead*) were observed rolling along the venous wall. Sequence of fluorescent fundus images 48 hours after LPS injection. Other *dots* are free-flowing leukocytes. T, number of seconds elapsed after administration of fluorescent platelets. **(C)** Time course of the number of rolling leukocytes after LPS injection. Values are mean ± SEM, $^{*}P < 0.01$, $^{\dagger}P < 0.05$, compared with control mice.

$P = 0.014$ vs. control mice). In veins (Fig. 1D), significant vasodilation occurred, peaked at 48 hours after reperfusion (150.4%, $P < 0.0001$ vs. control mice), and subsided at 72 hours after LPS injection.

There was no significant difference among groups in blood leukocyte counts. The peripheral leukocyte count was 1.0 ± 0.3 10^9/L in the control group. This became 1.1 ± 0.2, 1.6 ± 0.7, 1.4 ± 0.4, 2.0 ± 0.6, 1.8 ± 0.1, and 2.6 ± 0.6 × 10^9/L at 4, 12, 24, 48, 72, and 96 hours, respectively, after LPS injection.

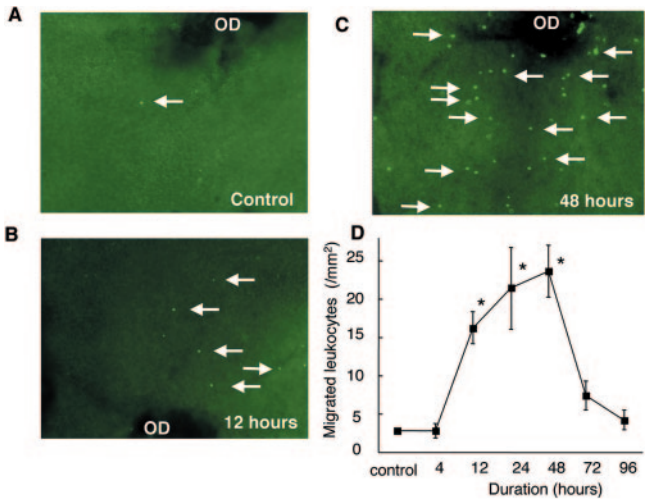

FIGURE 3. Leukocytes migrating in the retina were observed as fluorescent *dots* after AO injection in the microscopic image of whole-mounted retina (*white arrows*). OD, optic disc. A small number of leukocytes could be found in control mice **(A)**. Increasing numbers of leukocytes migrated at 12 hours after LPS injection **(B)** and peaked at 48 hours after LPS injection **(C)**. Time course of the number of leukocytes migrated in mouse retina after LPS injection **(D)**. Values are mean ± SEM, $^{*}P < 0.01$, compared with control mice.

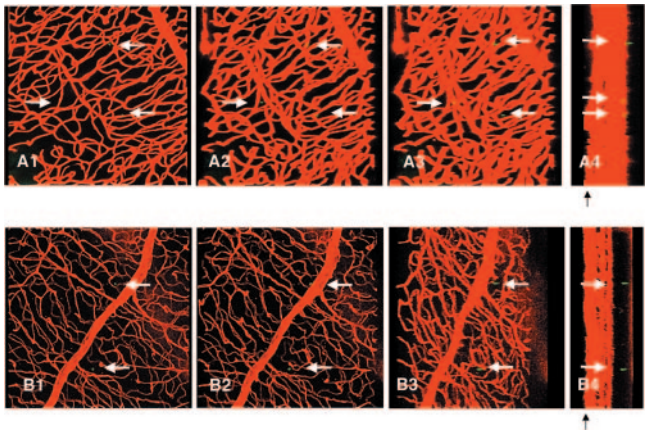

FIGURE 4. Sequence of the three-dimensional rotating retinal images of mouse retina 48 hours after LPS injection observed from various angles. In flatmounted retina obtained with a confocal microscope, the three-dimensional relationship of the leukocytes (stained with AO; *green dots*) and the vessels (stained with rhodamine-labeled Con A; *red*) were observed from various angles. *White arrows*, migrated leukocytes; *black arrows*, inner surface of the retina. The smaller *green dots without arrows* are artifacts that could be distinguished easily on the fluorescence microscopic observation. **(A)** In the retinal image 30 minutes after AO injection, the leukocytes just extravasated from the deeper capillary layers are clearly confirmed. **(B)** In the retinal image 10 hours after AO injection, the migrated leukocytes have moved toward the outer retina. **(A1)** and **(B1)**, front view of the retina; **(A2)**, **(A3)**, **(B2)** and **(B3)**, oblique view of the retina; **(A4)** and **(B4)**, cross-sectional view of the retina.

Leukocyte Rolling

Immediately after AO was infused intravenously, only leukocytes were stained among the circulating blood cells (Fig. 2A). Vascular endothelial cells also stained faintly. No rolling leukocytes were observed in the control group. In the mice with EIU, some leukocytes were observed slowly rolling along major retinal veins, but not along any major retinal arteries (Fig. 2B). At 4 hours after LPS injection, several leukocytes were observed rolling along the venous walls. At 12 hours after reperfusion, the flux of rolling leukocytes decreased slightly. The

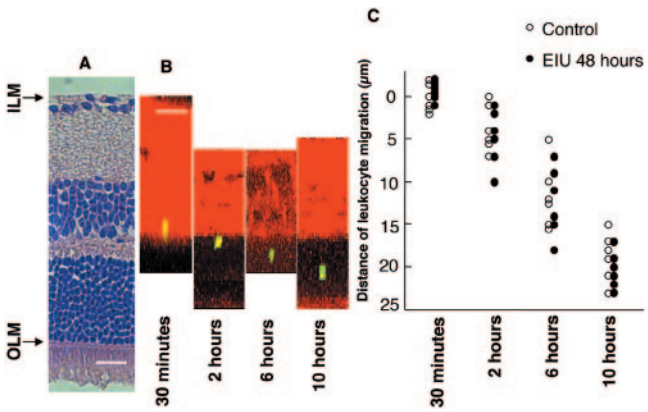

FIGURE 5. The migrated leukocytes travel toward the outer retina. **(A)** Histologic image of mouse retina 48 hours after LPS injection (stained with hematoxylin and eosin). **(B)** Time course of leukocyte migration after AO injection in mouse retina 48 hours after LPS injection. **(C)** Distance the migrated leukocytes traveled toward the outer retina from the deepest capillary layer. *Time*, time after AO injection; ILM, inner limiting membrane; OLM, outer limiting membrane. Scale bar, 20 μm; original magnification, ×200.

flux of rolling leukocytes then increased gradually and peaked at 48 hours after LPS injection (Fig. 2C).

Number of Migrated Leukocytes

At 30 minutes after AO injection, leukocytes in the vessels, as well as endothelial cells, became faint because of the washout effect, but migrated leukocytes in the retina could be identified as distinct fluorescent dots with the highest contrast (Fig. 3). We observed only those leukocytes that were exposed to a high concentration of AO in the vessels for a few minutes and that were extravasated before the washout effect. Figure 3D shows the numbers of leukocytes migrated in the retina at each time point. Few leukocytes were found in control retina (Fig. 3A). At 12 hours after LPS injection, the flux of migrated leukocytes began to increase (Fig. 3B). Flux of the migrated leukocytes peaked at 48 hours after LPS injection and increased to 8.4-fold of that of the control group (Fig. 3C; 23.7 ± 3.4 cells/mm^2).

Behavior of Migrated Leukocytes in Retina

In the three-dimensional image of flatmounted retina obtained with a confocal microscope, the relationship of the leukocytes (stained with AO; green dots) and the vessels (stained with rhodamine-labeled Con A; red) was observed from various angles (Fig. 4). In the retinal image obtained 30 minutes after AO injection, the leukocytes that had just extravasated from the deeper capillary layers were clearly seen (see Movies 1 and 2 at www.iovs.org/cgi/content/full/45/11/4197/DC1). As shown in Figure 5, all the migrated leukocytes traveled toward the outer retina 10 hours after AO injection. The migration velocity of the leukocytes was calculated to be 2.0 ± 0.1 μm/h at peak-time in the study group and 1.9 ± 0.1 μm/h in the control group ($P = 0.33$).

Discussion

This study demonstrated the behavior of migrating leukocytes in the retina, and followed the distribution of migrating leukocytes at select time points after AO injection (the beginning of the extravasation). The quantification of leukocyte migration into retinal tissue is useful to clarify the mechanism of retinal inflammation. More specifically, three new findings were obtained from this examination. First, leukocytes extravasated from the deeper capillary layers and not from the superficial capillary layer of the retina. Second, no leukocytes came from tissue other than the retina, such as choroid or vitreous. Third, all the leukocytes migrated from the deeper capillary layers toward the outer retina, and none of them emigrated from the retina into the vitreous space. These findings are important for further investigations of the role of leukocytes in the pathogenesis of inflammation.

Our first and second results are supported by an earlier study using immunochemistry conducted by Yang et al.[2] These authors reported that infiltration of macrophages into rat retina after LPS injection was seen only in the inner layers of the retina, whereas the photoreceptor layer was completely devoid of such cells. In addition, they suggested that there was no migration of macrophages into the retina from the choroid or the anterior segment. Our results agree with and strengthen their suggestions. Moreover, based on our third observation, the leukocytes that infiltrated into the vitreous were not derived from the retinal capillary layers and most likely come from anterior segments, such as the iris or ciliary body.

Several other methods are available to observe leukocyte migration.[16–20] To our knowledge, however, these other methods analyze leukocyte behavior only in plane fields or in an artificial experimental structure. With the current method, which uses a confocal microscope, clear three-dimensional images of leukocyte migration in the retina can be obtained and evaluated from various angles. This access is partly due to the transparency of the retina. Accordingly, our method may be valuable for detailed three-dimensional investigation in the analysis of leukocyte migration.

Using the method described herein, the velocity of leukocyte migration was calculated. In previous studies, the velocity of migrated leukocytes was measured to be approximately 6 μm/min in the collagen matrix model of Wolf et al.[17] and Friedl et al.,[18] 14 μm/min on the microslide of Luu et al.,[19] and 2–10 μm/h in the study of Palecek et al.[20] The velocity of leukocyte migration measured in our model was less than that in the collagen matrix or on the microslide. This finding may be due to the rich cellular components in the retina, such as Muller cells and horizontal cells, whereas no obstructing cells were encountered in the artificial experimental environment. Accordingly, the velocity in our study more closely reflects the physiological characteristics of the retina.

Moreover, we evaluated quantitatively the diameters of the major vessels, the number of rolling leukocytes, and the number of migrated leukocytes in mouse retina. These in vivo data are valuable to enable further understanding of the leukocyte-endothelial cell interactions that are regulated by various physiological factors, such as shear stress and chemical mediators. With the advances being made in genetic technology, the genetic expression of mice can now be easily modified. Consequently, using our current methods, we can quantitatively evaluate the genetic regulation of leukocyte–endothelial interactions in vivo.

In previous studies, our group evaluated leukocyte rolling in the major retinal vessels of the rat model of EIU. In the rat, the number of rolling leukocytes gradually increased and reached a maximum at 12 hours after LPS injection,[3,24] which is a little earlier than that in the mouse. This difference in peak times between the rat and mouse models may derive from the diversity of the species. The current methods can detect leukocytes labeled during the high-density phase of AO in the vessels, which lasts for only a few minutes. Accordingly, we can follow the changes in leukocyte–endothelial cell interactions more precisely as they occur.

In conclusion, our new methods have made it possible to evaluate quantitatively leukocyte–endothelial interactions and to follow leukocyte migration in mouse retina. Furthermore, the three-dimensional observation of leukocyte migration provides new information about leukocyte behavior. These innovative in vivo methods using mice may ultimately prove useful in the genetic investigation of microcirculation.

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