

## Investigation of the Source of the Blue Field Entoptic Phenomenon

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The cellular source of the blue field entoptic phenomenon was investigated in two microvascular preparations using video-microscopy with lighting conditions similar to those under which the entoptic phenomenon is visualized within the human eye. In the wing of the hibernating bat, microvascular flow was simultaneously videotaped under transmission illumination at 430 nm and under unfiltered illumination. In the rat cremaster alternating observations were made using transmission illumination at 430 nm and epi-illumination fluorescence microscopy with leukocytes rendered fluorescent by intravenous Quinacrine. In both preparations, low magnification video-microscopy using 430 nm illumination produced a field of particles, which were brighter than the background, flowing within a network of dark vessels. The appearance of the particles and their movement simulated the blue field entoptic particle motion. Under higher magnification, the particles appeared brighter than the plasma gaps between red blood cells and were demonstrated to be leukocytes by morphology, by specific staining and by typical behavioral movement. The particles were observed in terminal arteriolar capillaries, and post-capillary venules where they were not obscured by red blood cells. The results of this study of two microvascular preparations strongly suggest that in the human eye the blue field entoptic phenomenon is produced by leukocytes flowing within the macular retinal microvasculature. *Invest Ophthalmol Vis Sci* 30:668-673, 1989

Against a bright, diffuse illumination an entoptic phenomenon is observed of numerous bright particles that move in a flowing manner with synchronous rhythmic acceleration that corresponds to the cardiac cycle. The entoptic phenomenon has been thought to be produced by cells flowing within the macular retinal capillaries<sup>1</sup>; the movement is single file along recurrent paths without the passing or crossing of any two particles and is free and smooth with nonreversing motion. The particles seem to appear suddenly and flow along fixed, short, slightly curving paths before disappearing, which suggests that the entoptic phenomenon is produced by blood cells traversing the capillaries in front of the photoreceptors. The particles appear to most observers to skirt the center of fixation, leaving free an area that corresponds subjectively in size to that of the foveal avascular zone.

The cellular origin of the entoptically produced bright particles (which often show a dark tail) is controversial. Vierordt first suggested that the particle phenomenon was produced by leukocytes,<sup>2</sup> and this was supported by Bauermann who observed in leukemics that variations in the number of particles which passed repeatedly through one of the paths paralleled variations in the peripheral white blood cell count.<sup>3</sup> Observers in our laboratory have subjectively noted that the perceived particles commonly tend to appear at the start of the path during the acceleration phase of the movement, with completion of the movement within one cardiac cycle. This movement is consistent with leukocyte motion in capillaries.<sup>4</sup> Mean speeds of the entoptically perceived particles (0.75 mm/sec), which have been measured by Riva and Petrig with a simulation technique,<sup>5</sup> are similar to speeds of leukocytes measured in 7-10  $\mu$ m capillaries of the hamster cheek pouch,<sup>6</sup> rat cremaster,<sup>7</sup> and rabbit ear,<sup>4</sup> vessels of similar size to those of the retinal microcirculation.<sup>8</sup> Riva has suggested that leukocytes produce the bright entoptic image while traversing capillaries in front of the photoreceptors by interrupting the red blood cell column and transmitting light to the photoreceptors beneath.<sup>9</sup> The entoptic phenomenon is optimally perceived in the deep blue light spectrum at 430 nm, perhaps because hemoglobin has an absorption peak at this wavelength, which may produce relative dark adaptation of the

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photoreceptors lying beneath capillaries filled with red blood cells.

Wolbarsht et al, using an observation by Poisson that diffraction patterns for light passing through an opaque disc under certain conditions were similar to the diffraction patterns produced by an aperture of the same size, have suggested that the bright entoptic particles may be produced by subpopulations of red blood cell discs which flow through the capillaries with an orientation of the long axis perpendicular to the light path.<sup>10</sup>

The source of the entoptically perceived particles, whether produced by leukocytes, by red blood cells or by other plasma constituents, should be demonstrable by examining a microvascular preparation using the same transmission lighting conditions at 430 nm as those which produce the entoptic phenomenon in the human eye. We have examined the microvasculature in both the bat wing and rat cremaster muscle under such conditions in order to determine the cellular source of the blue field entoptic phenomenon.

### Materials and Methods

The investigations in this study using bats and rats conformed with the ARVO Resolution on the Use of Animals in Research.

#### Bat Wing Preparation

The wing of the hibernating *Myotis lucifugus* bat was prepared for video-microscopy as described by Wiedman et al.<sup>11</sup> The body of the bat was extended across the surface of a 2" × 6" microscope slide and held in place with spring clips. The unanesthetized bat exhibited no signs of distress. Two drops of mineral oil were placed between the under surface of the wing and slide in order to provide an illumination medium with less diffraction. The pigmented epidermal cells were teased away and the exposed microvasculature was covered with physiologic saline and a cover slip resting on slivers of glass to prevent compression of the tissue. Video-microscopy was conducted under conditions simulating blue field entoptoscopy. Under xenon illumination, the image was videotaped through one ocular of a Leitz ortholux binocular microscope with an interposed 430 nm interference filter (25 nm half band width) and was simultaneously videotaped through the other ocular without filters (white light). Microvascular flow was observed under two conditions: at low power (×100) to observe the microvascular network, and at high power (×740) to observe cellular detail within individual vessels. The videotapes of the individual vessels (in 430 nm light and in white light) were synchronized and viewed on separate monitors by several

independent observers who timed cellular passage as "events" using markers on a two-channel strip recorder. The monitor of the videotape taken under 430 nm illumination was defocused until no cellular morphology was observable, but the passage of a "bright flash" was marked as an event. On the monitor of the videotape taken under white illumination, the passage of a leukocyte, based on cellular morphology and typical behavioral movement, was marked.

Interobserver studies were conducted by comparing three independent observers who examined the 430 nm tape with three different observers who examined the tapes under white light. Each individual examined a videotape once only. Intraobserver correlation of the events in 430 nm and white light was conducted by having one observer examine both the 430 nm and white light video tapes each three times on separate occasions with sufficient time between tape examinations to prevent memory of event sequences.

#### Rat Cremaster Muscle Preparation

Male rats (8 weeks of age) were anesthetized with a single dose of sodium pentobarbital (5 mg/100 g, ip), placed on a heated mat, and the trachea cannula coupled to a Gould P23 ID pressure transducer. A femoral vein was cannulated for drug administration. The cremaster muscle was prepared for microscopic study using an adaptation of Baez's method.<sup>12</sup> Briefly, the cremaster muscle was separated from the scrotal sack and kept moist by a constant flow of Krebs solution maintained at 34°C. The solution was bubbled with a gas mixture of 95% N<sub>2</sub>-5% CO<sub>2</sub> to control pH at 7.40. The cremaster was spread over an optically clear, heated pedestal and the muscle temperature maintained at 34°C. The upper portion of the pedestal formed an open chamber that allowed the muscle to be superfused with Krebs solution (2 ml/min). Following these preparations the rat, already secured to a mounting board, was placed on the stage of a Leitz Laborlux 12 HL trinocular fluorescence microscope equipped with a 150 W xenon light source for transillumination and a 50 W ultra-high pressure mercury lamp epi-illumination for fluorescence imaging. The microscopic images were viewed directly and recorded using a closed-circuit TV system consisting of a low light level TV camera (MTI-65), video tape monitor (RCA, Model TC1119) and a video tape recorder (JVC, Model 6060U). The leukocytes, which are approximately 8 μm in diameter in the rat,<sup>13</sup> were rendered fluorescent by the intravenous administration of Quinacrine (1 mg/100 g). This dosage produces no demonstrable effect on systemic blood pressure or microvascular

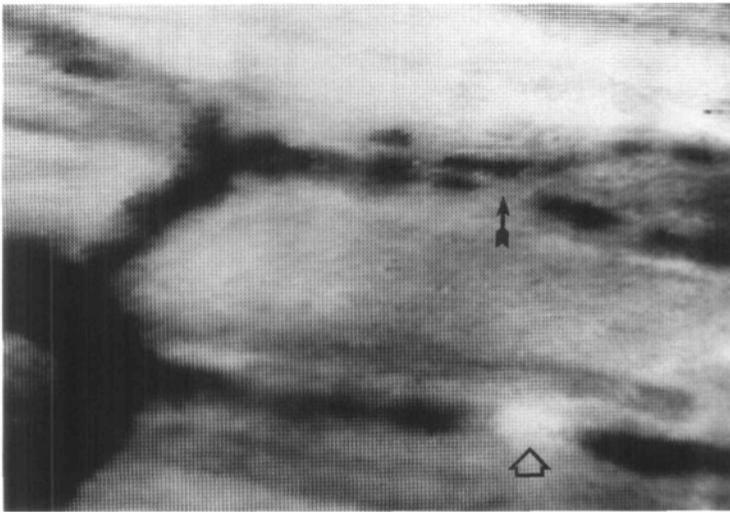


Fig. 1. Rat cremaster—transmission video-microscopy, 430 nm illumination. Two small post-capillary venules drain into a larger venule. A bright cellular image (open arrow) appears brighter than the plasma gaps between red blood cells (dark arrow).

flow. Quinacrine does not result in fluorescence of either erythrocytes or plasma components. Leukocyte dynamics were observed and recorded in microvessels ranging from 7–15  $\mu\text{m}$  diameter (diameters similar to those of the microvasculature in the human macula<sup>8</sup>). Magnification ranged from  $\times 50$  to  $\times 400$  using  $\times 10$  and  $\times 40$  water immersion objectives. Observations were made using both epi-illumination fluorescence and transillumination with a 430 nm interference filter placed in the light path between the transilluminating light source and the cremaster preparation.

Attempts were made simultaneously to perform fluorescence video-microscopy through one ocular of the microscope and 430 nm microscopy through the fellow ocular in order to analyze individual cell correlations. However, using standard fluorescence microscopy excitation filters in the illumination optics provided insufficient illumination to pass a 430 nm interference barrier filter for the 430 nm microscopy. On the other hand, using a 430 nm interference filter (25 nm half band width) for illumination provided too great an overlap with the fluorescence barrier filter, resulting in background pseudofluorescence that washed out observation of the individually stained cells. Therefore, 430 nm transmission microscopy and epi-illumination fluorescence microscopy were performed sequentially on the same vascular segments in alternating 1 min segments. The videotaped segments under fluorescence microscopy were reviewed by one observer independent of the reviewer who observed the segments under 430 nm

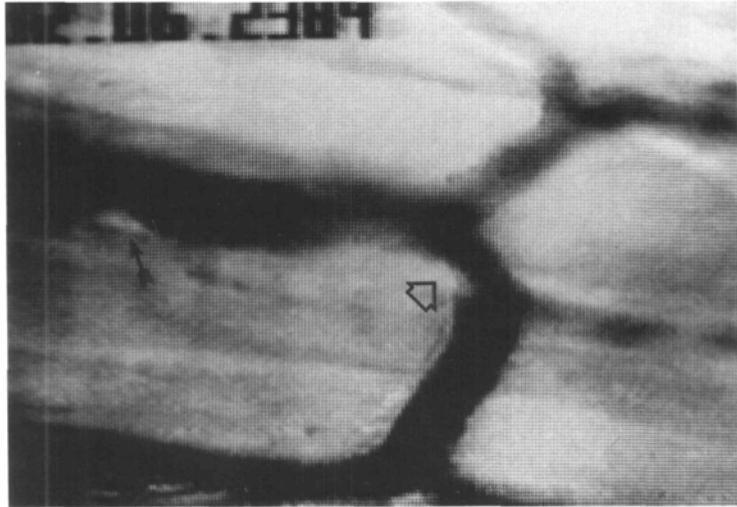
transillumination. Each observer marked the passage of a bright cell (or fluorescein-labeled cell) with an event marker on a strip chart recorder. The events were summed over the 1 min interval and were averaged for at least five intervals on each vascular segment.

### Results

In both the bat wing and rat cremaster muscle preparations low magnification video-microscopy at 430 nm produced a field of particles which were brighter than the background, flowing within a network of dark vessels. In both preparations, the field of bright particles and their movements simulated the blue field entoptic particle motion. Under higher magnification, the bright particles were observed in the terminal arteriols, capillaries, and post-capillary venules where they were not obscured by red blood cells and were observed to be brighter than the plasma spaces between cells (Fig. 1). In some instances the bright cells could be tracked through capillaries into larger venules where they were observed as bright spheres slowly rolling along the margins of the vessel (Fig. 2), movement typical of leukocytes.<sup>7</sup> In both preparations under higher magnification, the vessel walls and the plasma spaces between the dark red blood cells were subjectively observed to exhibit the same luminance as the background interstitium (Fig. 1). Vessels without red blood cells within the lumen (because of vasomotion) were invisible against the background.

Analysis in the rat cremaster of the sequential 1

**Fig. 2.** Rat cremaster—transmission video-microscopy, 430 nm illumination. A collecting venule is observed with a small bright cell (dark arrow) moving slowly along the wall, while a larger, bright cell (open arrow) is swept with flow around the curve to later adhere and roll along the wall.



min segments of 430 nm microscopy alternating with segments of fluorescence microscopy with leukocyte labeling are shown in Table 1 for four representative microvascular segments having diameters of 7–12  $\mu\text{m}$ . Analysis by student t-test of the numbers observed over five 1 min intervals for each vascular segment revealed no significant difference between the number observed under 430 nm conditions compared with the number observed under fluorescent light ( $P$  always greater than 0.2 for all vascular segments). On occasion in capillaries with very slow flow, the transillumination could be switched to fluorescence epi-illumination during the transit of a single cell. Under these circumstances, a bright cell passing under 430 nm light was always observed to be a fluorescein-labeled leukocyte.

For the bat wing preparation, in order to analyze the association between events recorded under 430 nm illumination and those obtained under white light, the strip chart segments were divided into uniform time intervals, such that events of cellular passage occurred in approximately 30% of the intervals (therefore reducing the chance to less than 9% that two cells would pass within one interval). The intervals varied for different vascular segments but averaged 2 seconds. A representative example is shown in Table 2 for a single observer reviewing the videotapes each three times for a 10  $\mu\text{m}$  vessel to evaluate single observer variability in cell identification. From Table 2, the estimated sensitivity for the detection of a passing leukocyte in white light was 0.96, while in 430 nm light the sensitivity was 0.90. The estimated specifi-

ties in white and 430 nm blue light were both 0.99 (please see Appendix for discussion of the calculations for sensitivity and specificity). The predictive value positive for a 30% event prevalence rate was 98% in both 430 nm and white light. The predictive value negative in white light was 98% and was 96% in 430 nm illumination. Table 3 demonstrates the results for three independent observers reviewing the videotape of a 10  $\mu\text{m}$  diameter vessel under 430 nm illumination while three separate observers reviewed the videotape of the same vascular segment under unfiltered illumination. From Table 3, the estimated sensitivity for the detection of a passing leukocyte is 0.84 in white light and 0.82 in 430 nm light, while the specificity is estimated in white light at 0.95 and in 430 nm light at 0.98. The predictive value positive (for a timed interval producing a 30% prevalence of events)

**Table 1.** Cellular passage in rat cremaster microvessels under 430 nm transmission video-microscopy and epi-illumination fluorescence video-microscopy\*

Vessel no.	Vessel diameter ( $\mu\text{m}$ )	430 nm microscopy† cells/min $\pm$ 1 SD	Fluorescence microscopy† cells/min $\pm$ 1 SD
1	10	11.5 $\pm$ 3.8	9.7 $\pm$ 3.7
2	12	16.6 $\pm$ 2.1	13.2 $\pm$ 4.3
3	7	1.7 $\pm$ .9	3.1 $\pm$ 1.5
4	10	6.8 $\pm$ 3.5	7.5 $\pm$ 3.1

\* Leukocytes are rendered fluorescent by IV quinicrine.

† The mean is an average for five intervals. Student t-test for each vessel demonstrates no significant difference between the cell fluxes observed under the two lighting conditions.

**Table 2.** Comparison of a single observer reviewing two transmission video-microscopy tapes simultaneously obtained of a 10  $\mu\text{m}$  diameter vessel under 430 nm illumination and under unfiltered illumination\*

		Number of intervals in which a leukocyte is identified under unfiltered illumination in three reviews of the videotape				
		0 of 3	1 of 3	2 of 3	3 of 3	Total intervals
Number of intervals under 430 nm illumination in which flash is observed on three reviews of videotape	0 of 3	431	5	2	3	441
	1 of 3	1	3	1	10	15
	2 of 3	1	2	5	15	23
	3 of 3	0	0	5	66	71
Total intervals		433	10	13	94	550

\* Each tape was reviewed three times. The videotapes were divided into a total of 550 intervals, each 2.0 seconds. In 431 of the intervals (upper left of table) no cells were observed in all three reviews of each videotape. In 66 of

the intervals (lower right of table) a cell passage was detected in all three reviews of each tape.

is 87% for white light and 94% for 430 nm light. The predictive value negative for both white and 430 nm light is 93%. Similar results were obtained for three other vessels measuring 6–12  $\mu\text{m}$ .

### Discussion

The results presented strongly suggest that the blue field entoptic phenomenon is produced by leukocytes traversing retinal capillaries that are in front of the photoreceptors, perpendicular to the light path. Transmission video-microscopy of two microvascular preparations using illumination at 430 nm demonstrated under low magnification a field of bright particles with movement characteristics strikingly similar to the entoptic phenomenon. Under higher magnification, to allow examination of individual vascular segments, the results strongly suggest the bright particles are produced by leukocytes and not

by subpopulations of red blood cells, as has been suggested by Wolbarsht et al,<sup>10</sup> since all of the RBCs appeared dark. The bright flashes were not associated with platelets or plasma gaps that occurred without leukocytes since these demonstrated no luminance qualities different from the background. The often-observed bright and dark patterns of the entoptic particles may be due to diffraction patterns produced by light passing through the leukocytes, however.

These results support the concept that the leukocyte produces an entoptically perceived bright flash as it traverses the capillary in front of the photoreceptor by interrupting the red blood cell column. The type of movement and the average length of the entoptic path<sup>5,15</sup> are in agreement with dimensions of the outer retinal capillary plexus (150  $\mu\text{m}$ ) as reported by Shimizu and Ujiie.<sup>8</sup> The demonstration that the blue field entoptic phenomenon may be produced by leukocytes which traverse the macular retinal capillaries

**Table 3.** Comparison of several observers reviewing two transmission video-microscopy tapes taken simultaneously of a 10  $\mu\text{m}$  diameter vessel under 430 nm illumination and under unfiltered illumination\*

		Number of intervals in which a leukocyte is identified under unfiltered illumination in three reviews of the videotape				
		0 of 3	1 of 3	2 of 3	3 of 3	Total intervals
Number of intervals under 430 nm illumination in which flash is observed on three reviews of videotape	0 of 3	125	17	5	1	148
	1 of 3	7	6	5	1	19
	2 of 3	1	3	17	18	39
	3 of 3	1	4	10	43	58
Total intervals		134	30	37	63	264

\* Each tape was reviewed by three different observers. The tapes were divided into a total of 264 intervals, each 2.0 seconds. In 125 of the intervals (upper left of table) none of the six observers detected a cell passage on either

the 430 nm or white light video tapes. In 43 of the intervals all six reviewers detected the passage of a cell.

provides a noninvasive means to measure retinal microvascular blood flow. Riva and Petrig have devised a simulation presented on a CRT screen which the observer compares and adjusts to match his own entoptic phenomenon.<sup>5</sup> The technique provides for a quantitative measurement of leukocyte density and of average leukocyte velocity and pulsatility. Measurement of the speed of an individual leukocyte as it passes through a capillary represents an excellent index of bulk blood flow rate within that capillary while the leukocyte is present,<sup>6,16</sup> although it may not be representative of flow when a leukocyte is not present. However, within a capillary network in which leukocytes are present simultaneously in a significant percentage of the vessels, the result will be to stabilize the flow variations within the network, and a good correlation should still remain between average leukocyte speed and average capillary blood flow. The velocities of leukocytes in the human retinal capillaries as evaluated by the blue field entoptic phenomenon are remarkably homogenous throughout the field and are measured simultaneously in hundreds of macular capillaries using the simulation technique. Therefore, the measurements represent an average indicator of bulk flow through these capillaries. Abnormalities of mean leukocyte velocity in disease may be interpreted as abnormalities of capillary blood speed and may also represent abnormalities of bulk blood flow, provided capillary diameter is not altered from normal. Physiologic alterations produced by moderate changes in perfusion pressure, or by changes in inspired oxygen or CO<sub>2</sub> may also be interpreted as alterations in microvascular flow since such physiologic maneuvers do not acutely alter capillary diameter.

**Key words:** retina, capillary, blood flow, entoptic, leukocyte

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## Appendix: Statistical Calculations

Specificity may be defined as the probability a leukocyte will not be observed in a single interval, given that the interval actually does not contain a leukocyte. Sensitivity, on the other hand, may be defined as the probability a leukocyte will be observed in a single interval, given that the interval actually does contain a leukocyte. (The length of each interval was chosen to make the number of intervals with multiple leukocytes negligible.) Both sensitivity and specificity will vary depending upon the type of light and the observer. Unfortunately, there is no way of knowing for certain whether an interval actually contains a leukocyte or not, because intervals are not always classified consistently by all observers and both false-positive and false-negative errors are possible.

Sensitivity, specificity and the proportion of intervals actually containing a leukocyte were therefore estimated simultaneously using a statistical model for the mixture of two binomial distributions.<sup>14</sup> Briefly, this model assumes for a single observation of a single interval, that: (1) all intervals which actually do not contain a leukocyte have the same probability (specificity) of being correctly classified; (2) all intervals which actually do contain a leukocyte have the same probability (sensitivity) of being correctly classified; and that (3) repeat observations on the same interval, or observations on different intervals are independent.

Estimates and standard errors were calculated using the method of maximum likelihood, without the necessity of knowing which intervals did or did not actually contain leukocytes. Separate analyses were done for the intra- and interobserver studies.