LPS-induced microvascular leukocytosis can be assessed by blue-field entoptic phenomenon

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Kolodjaschna, Julia, Fatmire Berisha, Solveig Lung, Georg Schaller, Elzbieta Polska, Bernd Jilma, Michael Wolzt, and Leopold Schmetterer. LPS-induced microvascular leukocytosis can be assessed by blue-field entoptic phenomenon. Am J Physiol Heart Circ Physiol 287: H691–H694, 2004. First published March 11, 2004; 10.1152/ajpheart.01240.2003.—Administration of low doses of Escherichia coli endotoxin (a lipopolysaccharide [LPS]) to humans enables the study of inflammatory mechanisms. The purpose of the present study was to investigate whether the blue-field entoptic technique may be used to quantify the increase in circulating leukocytes in the ocular microvasculature after LPS infusion. In addition, combined laser Doppler velocimetry and retinal vessel size measurement were used to study red blood cell movement. Twelve healthy male volunteers received 20 IU/kg iv LPS as a bolus infusion. Outcome parameters were measured at baseline and 4 h after LPS administration. In the first protocol (n = 6 subjects), ocular hemodynamic effects were assessed with the blue-field entoptic technique, the retinal vessel analyzer, and laser Doppler velocimetry. In the second protocol (n = 6 subjects), white blood cell (WBC) counts from peripheral blood samples and blue-field entoptic technique measurements were performed. LPS caused peripheral blood leukocytosis and increased WBC density in ocular microvessels (by 49%; P = 0.036) but did not change WBC velocity. In addition, retinal venous diameter was increased (by 9%; P = 0.008), but retinal blood cell velocity remained unchanged. The LPS-induced changes in retinal WBC density and leukocyte counts were significantly correlated (r = 0.87). The present study indicates that the blue-field entoptic technique can be used to assess microvascular leukocyte recruitment in vivo. In addition, our data indicate retinal venous dilation in response to endotoxin.

ocular blood flow; inflammation; retinal vessel analyzer; laser Doppler velocimetry

ADMINISTRATION OF SYSTEMIC doses of endotoxin is a unique means to study mechanisms of inflammation and has provided novel information regarding the interactions of inflammatory mediators and cell activation (5, 15). Polymorphonuclear leukocytes (PMNLs) play an important role in both host defense and systemic inflammatory responses. Vascular endothelium–PMNL interactions are considered to be critical reactions in the development of organ failure. Various reports have focused on endothelium–PMNL interactions and resultant endothelial injury: the production of cytokines and the expression of adhesion molecules in the vascular endothelium increases in the early phase of inflammation, and the release of cytokines and soluble adhesion molecules into the circulation correlates well with the degree of inflammation depending on the extent of the associated ischemia–reperfusion injury (14). The early phase of inflammation is characterized by leukocyte infiltration into tissues, especially neutrophils. This increase in circulating neutrophils is generally assessed by a white blood cell (WBC) count from a peripheral blood sample. Direct visualization of this phenomenon in the microvasculature has, however, not yet been reported.

The blue-field entoptic phenomenon represents a possibility to study leukocyte dynamics in vivo, because only red blood cells (RBCs)–and not WBCs–absorb short-wavelength light. When a subject looks into blue light, bright corpuscles are perceived as flying around his or her fovea. Hence, the passage of WBCs within the perimacular retinal capillaries is perceived as a flying corpuscle. A system that allows for the quantification of the blue-field entoptic phenomenon has been realized (13).

In the present study, we hypothesized that the blue-field entoptic technique may be used to quantify the increase of leukocytes and their flow behavior after lipopolysaccharide (LPS) infusion in the microvasculature. In addition, we used combined laser Doppler velocimetry and retinal vessel size measurement (12) to study RBC movement after LPS administration to assess retinal vasodilation during inflammation.

METHODS

Study design and subjects. Measurements were performed during two studies using the LPS model of inflammation. In protocol 1, the effects of LPS administration on ocular and systemic hemodynamics were measured. Retinal RBC and WBC dynamics were quantified in this protocol. In protocol 2, the effects of LPS on leukocyte changes in macro- and microvessels were studied. This was done by comparing retinal WBC flux with leukocyte counts from blood samples. The study was approved by the Ethics Committee of the Vienna Medical School, and all participants gave written informed consent before enrollment in the study. A total of 12 healthy male volunteers was recruited for the two protocols (n = 6 in each protocol). Mean ages of the study subjects were 27 ± 5 yr (n = 6) in protocol 1 and 27 ± 4 yr (n = 6) in protocol 2. Health status was determined by laboratory and clinical tests including medical history evaluations, physical and ocular examinations, and hematologic, biochemical, and virologic screenings. Exclusion criteria included hyperlipidemia, hyperglycemia, ametropia of >3 diopter, and regular or recent (within 3 wk) intake of any drugs. In all subjects, the right eye was studied.

Study protocol. The study started in the morning after an overnight fast. Subjects were rested for at least 20 min in a sitting position until stable hemodynamic conditions were obtained, which were ensured by repeated blood pressure measurements. In protocol 1, baseline readings were measured with the blue-field entoptic technique, the retinal vessel analyzer, and laser Doppler velocimetry. In protocol 2, a blood sample was drawn to determine total WBC count, and
measurements with the blue-field entoptic technique were done. All measurements were repeated 4 h after bolus administration of 20 IU/kg iv LPS (national reference endotoxin, Escherichia coli; U.S. Pharmacopeial Convention; Rockville, MD). Blood pressure values and pulse rates were monitored noninvasively with a Careview system (Hewlett-Packard; Palo Alto, CA). Intraocular pressure (IOP) values were measured by Goldmann applanation tonometry (Haag-Streit; Bern, Switzerland), and tympanic temperatures were recorded using an electronic thermometer (Thermoscan; San Diego, CA). Blood samples were collected by venipuncture into EDTA-anticoagulated Vacutainer tubes (Becton-Dickinson; Vienna, Austria) and were analyzed by routine laboratory methods.

Blue-field entoptic technique. This noninvasive method is described in detail by Riva and Petrig (13). We used a commercially available system for the quantification of retinal WBC movement (Oculix blue-field simulator; Oculix Sarl; Arbaz, Switzerland). For determinations of velocity and density of flowing corpuscles, a simulated particle field was shown to the subjects under study. By comparison with their own entoptic observation, subjects could adjust WBC density and mean flow velocity (WBC velocity). Retinal WBC flux was calculated as WBC flux = WBC density × WBC velocity. These outcome parameters characterize WBC dynamics in perimacular retinal capillaries in arbitrary units. In the present studies, at least five matching trials were performed by each subject. Only values with a coefficient of variation of <15% were considered accurate. Subjects who did not reach the required reproducibility were excluded from the study.

Retinal vessel analyzer. The retinal vessel analyzer (Imedos; Jena, Germany) is a commercially available system that comprises a fundus camera (FF 450; Carl Zeiss; Jena, Germany), a video camera, a real-time monitor, and a personal computer with analyzing software that provides accurate determinations of retinal arterial and venous diameters. Retinal vessel diameters are analyzed in real time with a maximum frequency of 50 Hz. For this purpose, the fundus is imaged onto the charge-coupled-device chip of the video camera. The consecutive fundus images are digitized using a frame grabber. In addition, the fundus image can be inspected on the real-time monitor and, if necessary, stored on a video recorder. Evaluation of the retinal vessel diameters can be performed either on- or off-line from the recorded videotapes. Because of the absorbance properties of hemoglobin, each blood vessel has a specific transmittance profile. Measurement of retinal vessel diameters is based on adaptive algorithms using these specific profiles. Whenever a specific vessel profile is recognized, the retinal vessel analyzer is able to follow this vessel as long as it appears within the measurement window. If the requirements for the assessment of retinal vessel diameters are no longer fulfilled, as occurs during blinking, the system automatically stops the measurement of vessel diameter. As soon as an adequate fundus image is achieved again, vessel-diameter measurement restarts automatically. To select a region of interest, the user defines a rectangle on the screen of the real-time monitor. This window can include a retinal artery, a retinal vein, or both. As long as the vessels under study are within the selected rectangle, the system automatically corrects for eye movements. This is again permitted by the adaptive nature of the diameter analysis. Hence, vessel diameter can be recorded as a function of time as well as a function of the position along the vessel. The system provides excellent reproducibility and sensitivity (9). In the present study, major temporal arteries and veins were studied. Measurements of the retinal venous diameters were taken between 1 and 2 disc diameters from the margin of the optic disc. RBC velocity was measured at the same locations by using bidirectional laser Doppler velocimetry.

Laser Doppler velocimetry and retinal blood flow: The principle of blood flow velocity measurement by laser Doppler velocimetry (model 4000, Oculix) is based on the optical Doppler effect. Laser light, which is scattered by moving particles (e.g., erythrocytes), is shifted in frequency. This frequency shift is proportional to the blood flow velocity in the retinal vessel. The maximum Doppler shift corresponds to the centerline erythrocyte velocity. With bidirectional laser Doppler velocimetry, the absolute velocity in the retinal vessels can be obtained (11). Retinal RBC flux was calculated based on these measurements of maximum erythrocyte velocity (Vmax) using laser Doppler velocimetry and the vessel diameters measured at the same location of the vessel (12). Mean blood flow velocity (RBC velocity) in retinal veins was calculated as \( \text{Vmax}/2 \) based on a parabolic velocity profile. RBC flux through a specific retinal vein \( i \) was then calculated as

\[
\text{RBC flux}_i = \left( \frac{\text{Vmax}_i}{2} \times (\pi \times d_i^2/4) \right)
\]

Applanation tonometry. IOP was measured by Goldmann applanation tonometry while subjects were under local anesthesia (1% fluoroscein-oxybuprocain; Croma Pharma; Vienna, Austria).

Statistical analysis. Statistical analysis was done with the CSS: Statistica software package (StatSoft; Tulsa, OK). Effects of LPS on the outcome parameters were assessed with paired t-tests. A \( P \) value of <0.05 was considered significant. For data description, values are given as means ± SD.

RESULTS

Systemic hemodynamic parameters are presented in Table 1. After LPS administration, expected mild and transient fluilike symptoms (fever, headache, myalgia, nausea) occurred in all subjects in both protocols. No severe, serious, or unexpected adverse effects were observed after LPS infusion. As shown in Table 1, administration of LPS increased pulse rate and tended to reduce blood pressure. The effects on systolic, diastolic, and mean arterial blood pressure values did not, however, reach the level of significance due to the limited number of subjects studied. LPS had no effect on IOP, which indicates that ocular perfusion pressure tended to decrease in parallel with systemic blood pressure.

Baseline values of the blue-field entoptic technique were comparable between the two study protocols (Table 2). In addition, the retinal vessel diameters, RBC velocity values, and RBC flux measurements were in the expected ranges.

In protocol 1, administration of endotoxin had no effect on WBC velocity (Fig. 1) but increased WBC density (\( P = 0.036 \)) and WBC flux (\( P = 0.035 \)). LPS increased venous diameter (\( P = 0.008 \)) but had no effects on RBC velocity or arterial

Table 1. Clinical and laboratory parameters at baseline and 4 hours after induction of systemic inflammation by Escherichia coli endotoxin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>4 h After Endotoxin Administration</th>
<th>( P ) Value</th>
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<tbody>
<tr>
<td>Temperature, °C</td>
<td>35.8 ± 0.5</td>
<td>37.1 ± 0.7</td>
<td>0.004</td>
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<tr>
<td>Pulse rate, beats/min</td>
<td>74 ± 11</td>
<td>97 ± 14</td>
<td>0.002</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>134 ± 18</td>
<td>129 ± 12</td>
<td>0.19</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>71 ± 11</td>
<td>66 ± 7</td>
<td>0.06</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>92 ± 12</td>
<td>87 ± 8</td>
<td>0.11</td>
</tr>
<tr>
<td>Intravascular pressure, mmHg</td>
<td>13.3 ± 2.9</td>
<td>13.0 ± 3.3</td>
<td>0.48</td>
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Table 2. Values are means ± SD; \( n = 6 \) subjects in each protocol. Escherichia coli endotoxin was administered as a 20 IU/kg iv bolus infusion.
diameter measurements. Accordingly, RBC flux increased \( (P < 0.024) \) but the effect was less pronounced than on WBC flux.

In protocol 2, the effects on blue-field entoptic parameters were similar to those observed in protocol 1 (Fig. 2). Again, the increase in WBC density was significant \( (P = 0.023) \), whereas the effect on WBC velocity was not. As also shown in Fig. 2, endotoxin caused the expected degree of leukocytosis \( (P = 0.003; \text{Ref. 3}) \). The changes in leukocyte count correlated with the changes in WBC density in the retina (Fig. 3).

**DISCUSSION**

Systemic administration of endotoxin is a well-established model of inflammation. LPS can cause intermittent flu-like symptoms such as fever, chills, myalgia, arthralgia, nausea, and headache. Overall, doses \( \leq 4 \, \text{ng/kg} \) have been well tolerated (15), and study subjects are virtually free from symptoms 8 h after LPS administration.

In the present study, we investigated retinal RBC and WBC movement after LPS administration in healthy subjects. The blue-field entoptic technique, laser Doppler velocimetry, and the retinal vessel analyzer were applied to assess these ocular hemodynamic parameters. The blue-field entoptic technique revealed a significant increase in the density of leukocytes in peripheral retinal capillaries. Leukocyte density but not velocity was increased in the subjects under study and was closely related to systemic leukocytosis. This is in agreement with a previous study from our group, which shows that the increase in circulating leukocytes in the human retina can be assessed after administration of granulocyte colony-stimulating factor by using the blue-field entoptic phenomenon (1). The results of

<table>
<thead>
<tr>
<th>Table 2. Ocular hemodynamic parameters at baseline</th>
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<tr>
<td><strong>Protocol 1</strong></td>
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<td>----------------</td>
</tr>
<tr>
<td>WBC velocity, au</td>
</tr>
<tr>
<td>WBC density, au</td>
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<tr>
<td>WBC flux, au</td>
</tr>
<tr>
<td>Arterial diameter, ( \mu \text{m} )</td>
</tr>
<tr>
<td>Venous diameter, ( \mu \text{m} )</td>
</tr>
<tr>
<td>RBC flux, ( \mu \text{min}^* )</td>
</tr>
</tbody>
</table>
| Values are means ± SD; \( n = 6 \) subjects in each protocol. WBC and RBC, white and red blood cell, respectively; au, arbitrary units. \( ^* \)RBC flux through one specific vein and not total RBC flux. Areas with no data indicate that parameters were not measured.

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Fig. 1. Effects of lipopolysaccharide (LPS) on ocular hemodynamic variables 4 h after administration (protocol 1; \( n = 6 \) subjects). Data are presented as percent change from baseline ± SD. WBCV, white blood cell (WBC) velocity; WBCD, WBC density; WBCF, WBC flux; AD and VD, arterial and venous diameter, respectively; RBCV, red blood cell (RBC) velocity; RBCF, RBC flux. \( ^*P < 0.05 \).

Fig. 2. Effects of LPS on variables assessed with the blue-field entoptic technique and leukocyte counts 4 h after LPS administration (protocol 2; \( n = 6 \) subjects). Data are means ± SD; a.u., arbitrary units.

Fig. 3. Linear correlation analysis of individual changes in WBC density in retinal capillaries and leukocyte counts in peripheral blood. Correlation line and the 95% confidence intervals are shown (protocol 2; \( n = 6 \) subjects).
the present study indicate that WBC density may be used to estimate changes in the number of circulating leukocytes in the retinal microvasculature. This is further supported by the fact that one low responder to LPS was identified with both methods: the peripheral leukocyte count and the blue-field entoptic technique. To the best of our knowledge, this is the first approach to visualize the increase in circulating leukocytes in a microvascular bed in a human model of inflammation in vivo.

Although a high degree of association was observed between the changes in leukocyte count and WBC density, a perfect correlation may not be expected. Blood drawn from an ante-cubital vein does not necessarily reflect leukocyte accumulation in different compartments; this is due to phenomena such as leukocyte margination, particularly in the microvessels. Moreover, the different sizes of neutrophils, lymphocytes, and granulocytes may result in different flowing and rolling properties in the retina and the forearm. These effects may be enhanced by differences in adhesion molecule expression in different beds of vasculature that are not accessible to measurement in the retina. Generally, it is assumed that selectin-mediated leukocyte rolling with subsequent integrin activation and integrin-mediated firm adhesion are responsible for recruitment. Interestingly, in liver and brain, other mechanisms appear to play a role as well (4). In brain, adhesion molecule expression is low, and platelets may have a significant role. In the retina, little is known about these mechanisms, and the blue-field entoptic technique may represent a unique tool to study these aspects in vivo.

This is of clinical importance, because uveitis is one of the most damaging ocular conditions in humans and can lead to edema, high IOP, and ultimately, destruction of the intraocular tissues and blindness. Experiments in animals injected with LPS have allowed the investigation of various ocular biological changes associated with inflammation. Endotoxin-induced uveitis is a generally used experimental model for uveitis that appears in Reiter’s syndrome, Crohn’s disease, ulcerative colitis, sarcoidosis, and Behçet’s disease. Although some aspects of leukocyte infiltration and rolling have been studied in this animal model (2, 16), our current knowledge on this topic in humans is still limited. Whether the blue-field entoptic method can be used to study retinal leukocyte behavior in patients with uveitis remains to be shown.

The present study also indicates an increase in RBC flux in the retina after LPS administration. This observation is most likely linked to increased retinal blood flow and retinal microvascular vasodilation. A variety of actions of LPS including activation of platelet function, the coagulation-fibrinolysis system, the prostaglandin system, and the complement system have been elicited and resulted in circulatory disturbances and especially in microvascular dysfunction. In humans, LPS produces a systemic hypotensive and inflammatory reaction in healthy subjects (15) with regional vasodilation of the forearm vasculature that is associated with impaired vascular response to adrenoceptor agonists. This profound and transient reduction in adrenoceptor-mediated constrictor was seen in the absence of inducible nitric oxide synthase expression in blood and was not associated with altered nitric oxide bioactivity (8). These findings suggest that altered adrenoceptor signal transduction is responsible for systemic vasodilation and hyporesponsiveness in the endothoxic model. Interestingly, in the brain, no change in cerebral blood flow (10) or even a decrease (7) in cerebral perfusion were reported during human endotoxemia despite systemic vasodilation. This indicates that vascular effects in the brain and retina are different, and further pharmacodynamic studies are required to study these differences in more detail. In addition, further studies are required to characterize the time course of retinal RBC and WBC flux after LPS administration in more detail.

In conclusion, the present study indicates that the blue-field entoptic technique is a useful approach to assess leukocyte recruitment in human inflammatory models in the retinal microvasculature. In addition, our data indicate retinal vasodilatation in response to endotoxin.

REFERENCES