Macrophages in the Retina of Normal Lewis Rats and Their Dynamics After Injection of Lipopolysaccharide

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Purpose. To investigate the density, distribution, and morphology of macrophages (bone marrow-derived microglia) and major histocompatibility complex (MHC) class II-positive cells in the retina of Lewis rats and the dynamics of these cells after systemic lipopolysaccharide (LPS) injection.

Methods. Immunohistochemistry was carried out using monoclonal antibodies specific to monocytes and macrophages (ED1, ED2) and MHC class II-positive cells (OX-6) on whole-mounts of the retina obtained from Lewis rats before and at different time points after footpad injection of 200 μg of LPS.

Results. The inner layers of the normal retina contained a network of macrophages, whereby ED1 and ED2 staining revealed similar results. Macrophages were either dendritiform or pleiomorphic in morphology, with the former predominant. The density of positive cells was higher at the peripheral part and the periequatorial part (271 ± 10 cells/mm² and 267 ± 9 cells/mm², respectively) than at the posterior part (196 ± 11 cells/mm²; P < 0.0001 in both cases). Lipopolysaccharide injection induced an early adherence of monocytes to retinal blood vessels, followed by a massive influx of the macrophages into the retina. The ED1–ED2 positive cells showed a variety of morphologic appearances: large round cells, pleiomorphic cells, and dendritiform cells. Pleiomorphic cells were striking at 48 hours, whereas dendritiform cells were predominant in the whole retina at 72 hours and thereafter. On day 14, the dendritiform cell numbers returned to approximately preinjection levels. Major histocompatibility class II-positive cells could not be found in the normal retina, nor after LPS injection.

Conclusions. The network of MHC class II-negative microglia in the retina were studied. These cells may play an important role in immunoregulation and stability of the immunologic microenvironment within the retina. Systemic LPS injection was followed by a massive influx of macrophages into the retina. The absence of MHC class II-positive cells in the retina after LPS challenge may be an important protective mechanism against possible autoimmune damage. Invest Ophthalmol Vis Sci. 1996;37:77–85.

Endotoxin, the lipopolysaccharide (LPS)-containing component of Gram-negative bacterial cell walls, is able to induce uveitis in a number of species, including rat,1–5 rabbit,4 and mice,5 when injected systemically. Endotoxin-induced uveitis (EIU) is characterized by the early breakdown of the blood–aqueous barrier and subsequently by acute inflammation of the anterior uvea.4,6 It has been found that polymorphonuclear cells, macrophages, and T lymphocytes are involved in EIU.1,2,5,7 Although a number of inflammatory mediators and cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 are implicated in the pathogenesis of EIU,8–11 the exact mechanisms are not elucidated completely.

The predominance of inflammation in iris and ciliary body in EIU has been well documented, enabling it to be a useful model for acute anterior uveitis seen in humans with such conditions as ankylosing spondylitis, Reiter’s syndrome, Crohn’s disease, ulcerative colitis, sarcoidosis, and Behcet’s disease.1,12 However, little is known about retinal involvement in EIU.

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In EIU induced in mice, some inflammatory cells were observed in the posterior vitreous body, but no inflammation was found in the retina or choroid. Inflammation in the retina of Lewis rats has been reported by some investigators using immunohistochemistry on tissue sections. This technique, however, does not allow a proper evaluation of the distribution and morphology of infiltrating cells nor of their relation to the blood vessels. There has been a breakthrough with the introduction of immunohistochemistry on flatmounts. Recently, this technique has been used for the evaluation of macrophages in the iris and ciliary body, but no reports have been published concerning the dynamics of retinal macrophages after LPS injection. In this article, we describe in detail the massive influx of macrophages into the retina after systemic administration of LPS as measured by immunohistochemistry on retinal wholemounts. Results emphasize retinal involvement during EIU and suggest that EIU may serve as a counterpart for generalized uveitis seen in humans. Another important finding in the study is the lack of major histocompatibility complex (MHC) class II-positive dendritic cells in both the normal and the inflamed retina, which may prevent the local presentation of autoantigens, thereby precluding secondary autoimmune attack.

**MATERIALS AND METHODS**

**Experimental Protocol**

Fifty-two inbred male Lewis rats (6 to 8 weeks of age), each weighing 150 to 200 g, were used in the study. All rats were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Lipopolysaccharide (Salmonella typhimurium; Difco, Detroit, MI) was dissolved in sterile pyrogen-free saline. 0.9% at a concentration of 2 mg/ml, and 50 μl was injected into each hind footpad of the Lewis rats, for a total dose of 200 μg LPS per animal. The rats were examined clinically with a slit-lamp microscope before and after LPS injection. At different time points—before LPS injection (normal group) and 4, 8, 16, 24, 48, 72, 96 hours, and 7, 10, and 14 days after LPS injection—retinal wholemounts were prepared as described below. Each group consisted of five Lewis rats, except for the normal group (eight rats) and the last two time points (two rats in each case).

**Isolation and Preparation of the Retinal Wholemounts**

Lewis rats were systemically anesthetized with an intramuscular injection of 50 μl of Hypnorm (Janssen Pharmaceutica, Goirle, Netherlands) and an intraperitoneal injection of 0.5 ml of sodium pentobarbital, and then heparinized with an injection of 0.3 ml of heparin (5000 IU/ml) into the left ventricle. Perfusion was then performed through the left ventricle with cold phosphate-buffered saline (PBS, pH 7.4) to expel all blood and hematogenous elements from the capillary bed. Eyes were enucleated and subsequently dissected behind the ciliary body into two parts, anterior and posterior. Lens, vitreous body (including vitreous membrane), and vitreous cortex were removed carefully from the posterior part. The retina was separated gently from the choroid and fixed in cold 100% ethanol for 5 minutes. Finally, the fixed retina was placed in PBS in a 24-well tissue culture dish and stored at 4°C until use.

**Immunohistochemistry**

Immunohistochemistry was performed on the retinal wholemounts according to the method described previously by McMenamin and coworkers. Monoclonal antibodies used were ED1 (recognizing a cytoplasmic antigen in rat monocytes, macrophages, and 90% of dendritic cells), ED2 (recognizing a membrane antigen on resident tissues macrophages, especially in connective tissues, and a small subpopulation in lymphoid tissues) (ED1 and ED2 were kindly provided by Dr C.D. Dijkstra, Free University, Amsterdam), and OX6 (recognizing rat MHC class II antigen) (OX6: Sera-Lab, Sussex, UK). A standard ABC technique using biotinylated sheep anti-mouse antibody (Amersham Life Science, Amersham, UK), streptavidin-biotin-peroxidase complex (DAKO, Glostrup, Denmark), and 3,3 diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) as the substrate, were used in the study. Incubation with the first antibodies at 4°C was performed overnight. All other procedures were carried out at room temperature. Endogenous peroxidase activity was eliminated by incubating the retina in PBS and 1% H2O2 for 20 minutes. Immunostained wholemounts of the retina were placed on slides with the inner side of the retina facing up, dried at room temperature, embedded in Entellan (Merck, Darmstadt, Germany), and covered.

**Quantitative Analysis**

Immunostained cells were counted using a calibrated eyepiece graticule under a microscope with ×25 objective lens. From periphery to optic nerve, the retina measured approximately 4 mm, and it was divided roughly into three parts: a peripheral part (approximately 1 mm in width), a periequatorial part (approximately 1 mm in width), and a posterior part (approximately 2 mm in width). Cells were counted in three separate fields in each part per wholemount, and the mean number of cells per rat was calculated. In total, eight rats were examined, and the mean ± SEM was determined for the whole group. Data were analyzed.
using Student’s t-test. Because of the uneven involvement of the retina at the early stage or because of too massive influx of positive cells at the full-blown stage of inflammation, quantitative analysis was performed only on normal retina, not on retinas after injection of LPS.

RESULTS

Clinical Manifestation

Ocular changes, such as cells and flare in the anterior chamber, were observed as early as 4 hours after LPS injection. All the rats manifested moderate-to-severe anterior uveitis at 16 hours and at 24 hours, as evidenced by cells in the anterior chamber, iris hyperemia, miosis, and hypopyon. Inflammation subsided gradually during the following time points and disappeared on day 7.

Density, Distribution, Morphology, and Location of Immunopositive Cells in the Retina of Normal Lewis Rats

A network of immunopositive cells was found in the retina of normal Lewis rats, whereby immunostaining with ED1 and ED2 revealed similar results. Macrophages (microglia) were dendritiform or pleomorphic in morphology, with the former representing more than 90% of the stained cells (Figs. 1, 2). The number of immunopositive cells in the peripheral part (271 ± 10 cells/mm²) and the periequatorial part (267 ± 9 cells/mm²) was significantly higher than in the posterior part (196 ± 11 cells/mm²; P < 0.0001). In some areas, dendritiform cells were distributed along the blood vessels within the retina.

Immunostaining with OX6 revealed that there were no detectable MHC class II-positive cells in the retina of normal Lewis rats. As a positive control for the staining of OX6 positive cells, we performed immunohistochemistry on iris wholemounts, which revealed results similar to those published by McMenamin and coworkers,15 and also on wholemounts of the choroid–sclera complex, which showed a network of MHC class II-positive cells in the choroid (Yang et al, manuscript in preparation), consistent with the results of Forrester et al.18

Retinal Changes After Lipopolysaccharide Injection

There was a dramatic change regarding the ED1- and ED2-positive cells in the retina after footpad injection of LPS (Figs. 1, 2). Typical changes in the retina are shown in Table 1. The changes began with a few ED1⁺ monocytes attaching to the retinal blood vessels 4 hours after LPS injection. Attachment of an increasing number of monocytes to the retinal blood vessels, accompanied by infiltration of some monocytes into the retina and occasionally by focal accumulation of these cells around the blood vessels, was the most striking feature at 16 hours (Fig. 1). Large numbers of monocytes emigrated to the retina at 24 hours. The whole retina was involved at 48 hours. At this time, most macrophages had a pleomorphic appearance, although numerous dendritiform cells also were found in the whole retina. At 72 hours, although there were still numerous pleomorphic and dendritiform cells, results were different from those found at 48 hours because there was a dramatic increase in dendritiform cells, whereas there was a decrease in pleomorphic cells. Most dendritiform cells had more and longer dendrites than seen in the normal retina before LPS injection. Results seen at 96 hours were, by and large, similar to those seen at 72 hours, although the number of pleomorphic cells further decreased. On days 7 and 10, the number of dendritiform cells gradually decreased. On day 14, the number of dendritiform cells and pleomorphic cells had almost returned to normal levels.

Morphologic changes of the monocytes were striking when they emigrated from the blood vessels to the retina. A continuous profile of morphologic transitions was obtained from careful observation of retinal wholemounts at different times. All the cells attaching to the blood vessels were small and round. The initial change, seen as early as 16 hours, was that some round cells began to enlarge when they appeared in the retina. Obvious changes were found between 24 and 48 hours after LPS injection. The small, round cells enlarged either into larger round cells or irregularly into pleomorphic cells. Some of the larger round cells appeared to be dying, as suggested by the enlarging of these cells and the presence of cellular debris. Part of the cellular infiltrate transformed into dendritiform cells, as evidenced by the appearance of fine dendrite-like branches. Newly formed dendritiform cells were different from those found in the normal retina: They stained more intensely with the ED1 and ED2 antibodies, were larger, and had longer and increased numbers of dendrites per cell. These dendritiform cells gradually decreased in size of cell body and number of dendrites. On day 14 after LPS injection, the appearance of the dendritiform cells was similar to that seen in the normal retina, although some of the cells still had more dendrites per cell. Major histocompatibility complex II-positive cells were not detected in the retina at any time after LPS injection.

DISCUSSION

To achieve its physiological function, the retina, like the brain, should have at its disposal a series of protective mechanisms against the invasion of pathogens and
FIGURE 1. Immunohistochemical staining of retinal wholemounts with monoclonal antibody ED1 (recognizing a cytoplasmic antigen in rat monocytes, macrophages, and 90% of dendritic cells). Wholemounts of the retina were obtained from normal Lewis rat (A) and at different time points after footpad lipopolysaccharide injection: 16 hours (B); 24 hours (C); 48 hours (D); 72 hours (E); 96 hours (F); day 7 (G); and day 14 (H). Magnification bar = 250 μm. The broken line in A, B, and G show blood vessels in the retina; blood vessels are oriented from left to right, appear lighter than the rest of the background, and are lined with macrophages (especially in A and B).

other various insults and challenges either from itself or from hematogenous elements and adjacent tissues. A network of macrophages documented here is consistent with the results reported by Perry et al.19-21 who presented immunohistochemical evidence of microglia in the outer plexiform layer of the mouse retina using the F4/80 monoclonal antibody. These cells are able to provide the retina with an important protective mechanism. It is well established that macrophages are critical for host defense against viral, bacterial, and parasitic infection and for immunoregulatory function (either by presenting antigens to lymphocytes or by secreting a number of active substances and cytokines, including IL-1, IL-6, IL-8, TNF-α, and TGF-β).9,22,23 In addition, macrophages play an important role in tissue repair by elaboration of elastase and collagenase, and in the destruction of tumor cells.24 Furthermore, studies by Perry and coworkers19,20,21 have suggested that macrophages possibly exert a function in the development and regeneration of nerve tissue and are necessary both for the catabolism of neurotransmitters and hormones and for the lipid

FIGURE 2. Immunohistochemical staining of retinal wholemounts with monoclonal antibody ED2. Wholemounts of the retina were obtained from normal Lewis rat (A) and at different time points after footpad lipopolysaccharide injection: 48 hours (B); 96 hours (C); and day 14 (D). Magnification bar = 250 μm.
wholemount technique has advantages over general wholemount technique used in this study. The functions are necessary because of the variety of physiological events occurring in the retinal microenvironment and because the retina is exposed to insults from light, metabolic, hematogenous, and immunologic origin.

The demonstration of a network of macrophages in the normal retina is established readily with the wholemount technique used in this study. The wholemount technique has advantages over general tissue sectioning in that it can provide good visualization of the distribution pattern and morphology of the immunocompetent cells in the normal and inflamed retina. Additionally, the dynamics both in number of cells and in morphologic transition can be easily evaluated with this technique. Until now, the technique has been used by others to investigate macrophages in the normal iris and the iris after LPS injection.26

One of the important steps in the study of immunopositive cells within tissues using wholemounts is to exclude possible interference from another origin. To study monocytes and macrophages, blood should be removed completely by whole body perfusion. Another important step is to ensure that cell markers are preserved optimally. For this purpose, others have used perfusion and fixation with cold 100% ethanol and, subsequently, difficult to manipulate. Therefore, we used a modified procedure in this study. Perfusion only with cold PBS allowed us to manipulate the retina easily, which was placed into cold 100% ethanol rapidly after isolation to preserve antigenicity of the tissue.

There are two kinds of microglia in the central nervous system: perivascular microglia and parenchymal microglia. The former originates from a bone marrow precursor and expresses the marker ED2, whereas the origin of the latter population is still uncertain.26 The cells identified with ED1 and ED2 in the retina, as described in our study, are most probably the bone marrow-derived microglia. The exact distribution of non-bone marrow-derived microglia in the retina may be elucidated using a double staining technique with markers such as OX42, which recognizes the complement receptor type 3 present on monocytes–macrophages and microglia.27

Our study reveals that ED1- or ED2-positive cells are distributed in the whole retina of Lewis rats. In the study of McMenamin and coworkers,25 only OX42-positive cells were found in the peripheral retina, whereas no ED2-positive cells were noted in this area. This discrepancy may be attributed to the fact that these authors based their observations on frozen sections.

Inflammation induced by LPS has been an area of intensive study during recent years. Studies by Andersson and coworkers26 have revealed severe changes in the central nervous system parenchyma and a distinct inflammatory pattern after LPS challenge. The involvement of the retina after LPS injection presented here is not surprising because it is an extension of the brain. Our study provides firm evidence to indicate that the retina is involved intensely during EIU, a finding already noted by others using tissue sections.13,14 However, the importance of the retinal involvement in EIU has not been recognized widely. Past neglect concerning retinal involvement may be attributed to several factors: Unlike iris and ciliary body, the retina cannot be evaluated clinically, especially at the stage of full-blown inflammation of the anterior segment; experiments with ocular sections do not provide a whole profile of retinal involvement during EIU; different preparations of endotoxin are used, and some only induce mild inflammation. Immunohistochemical study of retinal wholemounts elucidates the importance of retinal involvement in EIU. Current data, together with the observation that the choroid was involved intensely during EIU (Yang et al, manuscript in preparation), provides a deeper understanding concerning the EIU model. It may serve hypothetically as a model for acute anterior uveitis and for generalized uveitis in humans.

Retinal changes began 4 hours after LPS injection, as evidenced by the adherence of monocytes to retinal blood vessel walls. At 16 hours, large numbers of monocytes attached to the blood vessels, and, in some

### TABLE 1. Main Features of Macrophages in the Retina at Different Time Points After Systemic Lipopolysaccharide Injection

<table>
<thead>
<tr>
<th>Time</th>
<th>Features</th>
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<tbody>
<tr>
<td>4, 8, 16 hours</td>
<td>Round cells (monocytes) adhered to blood vessels and began to emigrate into the retina.</td>
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<tr>
<td>24 hours</td>
<td>Large numbers of round cells (monocytes) were present in most of the retina. Round cells started changing shape into either pleomorphic or dendritiform cells.</td>
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<tr>
<td>48 hours</td>
<td>Involvement of the whole retina. Both pleomorphic cells and dendritiform cells increased in number.</td>
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<tr>
<td>72, 96 hours</td>
<td>A large number of dendritiform cells with more and longer dendrites was seen in the whole retina. Some cells appeared to be dying and disintegrated.</td>
</tr>
<tr>
<td>7, 10, 14 days</td>
<td>Cell numbers of the dendritiform cells gradually decreased and returned to the normal levels. Some dendritiform cells still possessed more and longer dendrites.</td>
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areas, these cells migrated to tissue and accumulated around the blood vessels involved. Subsequently, local involvement changed to generalized retinal inflammatory response. Longitudinal changes observed at the early stage of EIU provide direct evidence to demonstrate that the immunopositive cells seen in the retina originate from retinal blood vessels rather than from adjacent tissues. In addition, in experiments using cryostat sections of the eyes, we found that only the inner layers of the retina were infiltrated with macrophages, whereas the photoreceptor layer was completely devoid of these cells (data not shown), indicating that no migration occurs from the choroid to the retina. There is no evidence suggesting earlier and more severe involvement in the peripheral retina adjacent to the ciliary body, which makes it unlikely that the whole involvement of the retina is caused by spillover or spreading from inflammation from the anterior segment. Hence, retinal changes undoubtedly occur as a result of a direct response of the retina to LPS rather than as an epiphenomenon of the inflammatory response in the anterior segment.

There are, however, differences between the anterior uvea and the retina concerning the time course of cell infiltration after LPS injection. Retinal changes peak at approximately 48 hours, whereas inflammation in the anterior segment is most severe at 24 hours (Yang P, unpublished observations, 1995), a finding observed by others using tissue sections1-3 and wholemount studies of iris and ciliary body.29 Various factors may be responsible for the difference in time kinetics. Rich, fenestrated capillary beds with high blood flow rates in the ciliary body may respond more quickly than nonfenestrated vessels in the retina, thereby leading to the early peak of inflammation in the anterior uvea.

Recent studies by McMenamin and coworkers15,25 have revealed that there is a rich network of MHC class II+ dendritic cells and macrophages in the iris and ciliary body. These cells are presumed to serve as a potential source of cytokines on exposure to circulating endotoxin, leading to an amplification of the inflammatory response. The difference in the density of macrophages between the anterior uvea (approximately 700 cells/mm²)15,25 and the retina (approximately 270 cells/mm² in the peripheral and periequatorial parts) also may be involved in the delay of inflammation in the retina after LPS administration.

One of the interesting findings in this study is the absence of MHC class II-positive cells in the normal retina. It has been speculated that the brain is immunologically privileged, partly because of the absence of MHC class II-positive cells in gray matter.31 Similarly, the retina has been thought of as an immunologically privileged site with few MHC class II-positive cells.32,33 Our study is consistent with these findings. Using the same reagents and immunohistochemistry technique, we have demonstrated the presence of MHC class II-positive cells in the iris and choroid, thus proving the validity of the reagents used in this study. Because there are a number of uveitogenic antigens in the retina, MHC class II-negative macrophages may play a pivotal role in immunoregulation within the retina. Major histocompatibility complex class II antigens are necessary for the presentation of antigen by antigen-presenting cells to helper T lymphocytes.33,34 It has been proven that retinal S-antigen, interphotoreceptor retinoid-binding protein, rhodopsin, and so on, induce experimental autoimmune uveoretinitis and that these antigens have been implicated in some clinical forms of uveitis.35,36 These uveitogenic antigens may not be presented to potentially autoreactive T lymphocytes under normal conditions because of the absence of MHC class II-positive antigen-presenting cells, thus preserving the retina from autoimmune reaction damage.

The expression of MHC class II antigens on macrophages and microglia in the central nervous system after LPS challenge has been noted by Andersson et al30 and Montero-Menei et al.27 However, no MHC class II-positive cells could be detected in the retina after LPS injection, which may be of importance. Damage to the retina, especially to photoreceptors, may lead to the exposure of uveitogenic antigens to neighboring cells. If MHC class II-positive cells exist, an autoimmune response may ensue. Speculation has it that this is one of the mechanisms for the initiation and perpetuation of ocular inflammation in humans.37,38 Lack of MHC class II-positive cells after LPS injection may be one reason for short-lived ocular inflammation. Absence of mRNA for interferon-γ in the retina during EIU may be involved in the lack of MHC class II antigen expression because interferon-γ has been proven to be a potent inducer of MHC antigen expression.39,40 Whether immunization of rats with retinal antigens in the presence of complete Freund’s adjuvant and pertussis toxin leads to an induction of MHC class II positive antigen-presenting cells is an intriguing question that may be answered easily by using the retinal flatmount technique described in this article.

Key Words
immunohistochemistry, lipopolysaccharide, macrophages, retinal wholemounts, uveitis

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References


Macrophages in the Retina


