Pathogenic Role of Retinal Microglia in Experimental Uveoretinitis

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PURPOSE. To devise methods for unequivocal identification of activated retinal microglia in experimental autoimmune uveoretinitis (EAU) and to investigate their role in the development of EAU.

METHODS. A group of Lewis rats underwent optic nerve axotomy with the application of N-(4-(4-didecylaminostyryl)-N methylpyridinium iodide (4Di-10ASP) at the axotomy site. On days 3, 14, and 38 after axotomy, the rats were killed, the eyes were enucleated, and the retinas were stained for OX42. Another group of such axotomized rats were immunized with S-antigen peptide and were killed on days 7 through 12 after the injection with peptide. The enucleated eyes were stained for OX42 and examined by confocal microscope. After axotomy, bone marrow (Y3) chimeric rats were injected with S-antigen peptide and were killed on days 10 and 12 after injection. The retinas were evaluated by PCR with Y-specific primers. Finally, a group of axotomized rats was injected with the S-antigen peptide and killed on days 6, 8, 9, and 10 after injection. Their enucleated eyes were examined for microglial expression of TNFα and for generation of peroxynitrite.

RESULTS. In the axotomized, non-EAU eyes, 4Di-10ASP-labeled ganglion cells were detectable on days 3 and 14, and 4Di-10ASP-containing OX42-positive cells (microglia) were found in the nerve fiber and other inner retinal layers on days 14 and 38. The S-antigen peptide–injected rats showed migration of the microglia (4Di-10ASP–positive and OX42-positive) to the photoreceptor cell layer on day 9, and these cells increased in number at this site on day 10. No macrophages (OX42-positive and 4Di-10ASP-negative) were present at this early stage of EAU, but such cells appeared in the retina on days 11 and 12. PCR of the chimeric EAU retinas showed an absence of the Y chromosome–amplified product on day 10, but the presence of this product was detected on day 12. The expression of TNFα and generation of peroxynitrite were noted in the migrated microglia at the photoreceptor cell layer on days 9 and 10 of EAU.

CONCLUSIONS. In the early phase of EAU, the microglia migrate to the photoreceptor cell layer where they generate TNFα and peroxynitrite. Such microglial migration and activation take place before infiltration of the macrophages. These findings indicate a novel pathogenic mechanism of EAU, in which retinal microglia may initiate retinitis with subsequent recruitment of circulation-derived phagocytes, leading to the amplification of uveoretinitis. (Invest Ophthalmol Vis Sci. 2003;44: 22–31) DOI:10.1167/iovs.02-0199

S-antigen–induced uveoretinitis, commonly known as experimental autoimmune uveoretinitis (EAU), is characterized by the initial infiltration of mononuclear cells in the retinal perivascular sites, followed by the infiltration of phagocytes in the outer retina and uveal tract.1,2 Such infiltration is followed by retinal damage, which is thought to be caused by circulation–derived phagocytes through the release of cytotoxic oxygen and nitrogen metabolites, cytokines, and proteolytic enzymes.1–3 Although the retinal perivascular site is richly endowed with microglia that exhibit functions similar to the macrophages, the role of resident microglia in the initiation or amplification of EAU has not been determined.4

It has recently been proposed that microglia play an active proinflammatory role in the central nervous system (CNS) in several experimental and clinical diseases, including experimental autoimmune encephalomyelitis, cerebrovascular disease, Alzheimer’s disease, and multiple sclerosis.5 However, the identification of activated microglia in these pathologic conditions has been hampered by a lack of precise markers that can unambiguously distinguish the microglia from circulation–derived macrophages.6

In a normal retina, microglia can be distinguished from macrophages primarily by morphologic features and perivascular distribution.6 However, when activated, as in pathologic conditions, microglia lose their highly branched morphology, acquire short processes, and become hypertrophied.6–8 These activated microglia share virtually all the morphologic features of infiltrating macrophages, including several cell surface markers, including ED1, -2, and -3; OX42; phosphotyrosine; and integrin B4.7 Depending on the species of the animal and the state of microglial activation, some of these markers can probably distinguish microglia from macrophages.9–11 However, no such markers can distinguish activated microglia from macrophages.7 Retinal microglia are activated in EAU. Because no surface or other molecules can be used to specifically identify retinal microglia in EAU, a combination of multiple techniques is needed to distinguish the activated retinal microglia from macrophages.

In recent years, Thanos and Richter1,2 have reported a novel method in which the fluorescent dye N-4-(4-didecylaminostyryl)-N methylpyridinium iodide (4Di-10ASP; Molecular Probes, Eugene, OR) is used to detect retinal microglia after optic nerve axotomy. The dye initially appears in the retinal ganglion cells through retrograde axoplasmic transport. It is then taken up by the retinal microglia as a result of their phagocytosis of the dye-labeled ganglion cells. The retinal microglia containing this lipophilic dye remain labeled for up to 12 months.12 This type of long-term labeling may offer an opportunity for evaluating the role of retinal microglia in the initiation and perpetuation of EAU. However, the influx of circulation–derived monocyte-macrophages in EAU could phagocytose the degenerating dye-containing ganglion cells. Therefore this method

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22
per se may not unequivocally distinguish microglia from macrophages.

The male-to-female (Y→X) chimeric construction and the detection of Y chromosome-specific probe may allow for the precise differentiation of male bone marrow-derived cells. A rat cDNA probe specific for the Y chromosome has recently been reported. The use of bone marrow-chimeric rats with optic nerve axotomy, combined with the application of 4Di-10ASP may allow for the precise detection and localization of activated retinal microglia, and it may also facilitate differentiation of these native retinal cells from circulation-derived macrophages. Such differentiation is crucial for evaluating the role of microglia in EAU.

The purpose of this study was to devise methods that unequivocally identify the activated resident retinal microglia in EAU—especially, to distinguish them from the infiltrating macrophages—and to discern the role of resident microglia in the development of this intraocular inflammation. For this purpose, EAU was induced in chimeric (Y→X) rats after optic nerve axotomy. The mobility of microglia, as well as the arrival of hematogenous macrophages, was tracked sequentially, using confocal microscopy and polymerase chain reaction (PCR) to detect the Y chromosome. The pathogenic role of migrated microglia was assessed in vivo by detecting the generation of proinflammatory nitrogen species (peroxynitrite) and the expression of tumor necrosis factor-α (TNF-α).

**METHODS**

Lewis rats weighing 150 to 175 g (Charles River Laboratory, Wilmington, MA) were used in all the studies. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**In Vivo Labeling of Retinal Microglia**

Six female Lewis rats were anesthetized, and a lateral canthotomy was performed on them, followed by a superior conjunctival peritomy. Dissection was performed posteriorly until the retrolubar optic nerve was identified. A slit was created in the optic nerve sheath, parallel to the direction of the nerve fibers. Scissors were used to transect the optic nerve within the sheath, and 4Di-10ASP was packed at the cut surface of the optic nerve. The lateral canthotomy was then sutured. Axotomy was performed on right eyes only. To examine the retina in wholemount, three rats each were killed on postaxotomy days 3 and 14. The enucleated globes were fixed in phosphate-buffered 4% paraformaldehyde at 4°C for 4 hours. The globes were sectioned to discard the anterior segment and vitreous. The posterior segments were radially divided into four segments, and the retinas were separated from the choroids and incubated in 1% Triton X-100 in phosphate-buffered saline (PBS) for 1 hour and then washed in PBS. The retinas were incubated at 4°C overnight with mouse anti-rat monoclonal antibody (Santa Cruz, Santa Cruz, CA) at room temperature for 3 hours. The entire retina was flat-mounted and the 4Di-10ASP and Texas red were visualized by confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany) with excitation and emission set at 488 and 530 nm, respectively, for 4Di-10ASP and 543 and 610 nm, respectively, for Texas red.

**Migration of Retinal Microglia in EAU**

Thirty-two chimeric (Y→X) rats were constructed by using sterile techniques. The bone marrow removed from the tibia, femur, and humerus of the male donor rats was passed through a density gradient medium (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Piscataway, NJ) to remove red blood cells and cell debris. The cells were washed with RPMI 1640 (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum. Viability of the nucleated bone marrow cells was greater than 95%, as determined by trypan blue. The female rats were irradiated with 1000 rads by a cesium-135 gamma irradiator (Gamma cellulose rat spleen was subjected to PCR amplification with the following pair of primers: 5′-CATCAGGATTGATGCCAAT-3′ (5′ primer) and 5′-ATAGTGTAGTGGTTGGTCC-3′ (3′ primer). The amplified product (Y-104 bp) was confirmed by agarose gel electrophoresis, with a 100-bp DNA ladder used as a molecular weight marker. Cloning was performed according to the method described by Sambrook et al. Briefly, PCR-amplified Y-104-bp fragments were directly ligated to the T overhangs of vectors (pGEM; Promega, Madison, WI). The DH52 CaCl2-competent cells were transfected with circular plasmids. The recombinants were screened using the blue-white selection procedure. Three white colonies were randomly picked up and cultured. Plasmid DNA was then isolated and subjected to restriction enzyme digestion and agarose electrophoresis, with a 100-bp DNA ladder used as a molecular weight standard. The inserts were sequenced according to the method described by Sanger et al. To determine the specificity and sensitivity of the Y-104-bp fragments for male DNA, male-female splenic DNA mixtures were prepared in the ratio ranging from 1:1000 to 1:100 of male-to-female DNA (in nanograms). A similar experiment for assessing the limit of PCR detection was performed, with male-female spleen cells also mixed in the range of 1:10,000 to 1:100. These mixtures were amplified for 35 cycles using primers for a Y-104-bp fragment.

**Construction of Chimeric Rats and Determination of Stability of the Retinal Microglia Compartment**

Thirty-two chimeric (Y→X) rats were constructed by using sterile techniques. The bone marrow removed from the tibia, femur, and humerus of the male donor rats was passed through a density gradient medium (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Piscataway, NJ) to remove red blood cells and cell debris. The cells were washed with RPMI 1640 (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum. Viability of the nucleated bone marrow cells was greater than 95%, as determined by trypan blue. The female rats were irradiated with 1000 rads by a cesium-135 gamma irradiator (Gamma cellulose rat spleen was subjected to PCR amplification with the following pair of primers: 5′-CATCAGGATTGATGCCAAT-3′ (5′ primer) and 5′-ATAGTGTAGTGGTTGGTCC-3′ (3′ primer). The amplified product (Y-104 bp) was confirmed by agarose gel electrophoresis, with a 100-bp DNA ladder used as a molecular weight marker. Cloning was performed according to the method described by Sambrook et al. Briefly, PCR-amplified Y-104-bp fragments were directly ligated to the T overhangs of vectors (pGEM; Promega, Madison, WI). The DH52 CaCl2-competent cells were transfected with circular plasmids. The recombinants were screened using the blue-white selection procedure. Three white colonies were randomly picked up and cultured. Plasmid DNA was then isolated and subjected to restriction enzyme digestion and agarose electrophoresis, with a 100-bp DNA ladder used as a molecular weight standard. The inserts were sequenced according to the method described by Sanger et al. To determine the specificity and sensitivity of the Y-104-bp fragments for male DNA, male-female splenic DNA mixtures were prepared in the ratio ranging from 1:1000 to 1:100 of male-to-female DNA (in nanograms). A similar experiment for assessing the limit of PCR detection was performed, with male-female spleen cells also mixed in the range of 1:10,000 to 1:100. These mixtures were amplified for 35 cycles using primers for a Y-104-bp fragment.

**Detection of Y Chromosome–Containing Cells by PCR**

A 104-bp probe was generated from the genomic DNA of male rats using primers selected to the murine Sry gene, the sex-determining region of the Y chromosome. Briefly, genomic DNA from male rat spleen was subjected to PCR amplification with the following pair of primers: 5′-CATCAGGATTGATGCCAAT-3′ (5′ primer) and 5′-ATAGTGTAGTGGTTGGTCC-3′ (3′ primer). The amplified product (Y-104 bp) was confirmed by agarose gel electrophoresis, with a 100-bp DNA ladder used as a molecular weight marker. Cloning was performed according to the method described by Sambrook et al. Briefly, PCR-amplified Y-104-bp fragments were directly ligated to the T overhangs of vectors (pGEM; Promega, Madison, WI). The DH52 CaCl2-competent cells were transfected with circular plasmids. The recombinants were screened using the blue-white selection procedure. Three white colonies were randomly picked up and cultured. Plasmid DNA was then isolated and subjected to restriction enzyme digestion and agarose electrophoresis, with a 100-bp DNA ladder used as a molecular weight standard. The inserts were sequenced according to the method described by Sanger et al. To determine the specificity and sensitivity of the Y-104-bp fragments for male DNA, male-female splenic DNA mixtures were prepared in the ratio ranging from 1:1000 to 1:100 of male-to-female DNA (in nanograms). A similar experiment for assessing the limit of PCR detection was performed, with male-female spleen cells also mixed in the range of 1:10,000 to 1:100. These mixtures were amplified for 35 cycles using primers for a Y-104-bp fragment.

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from this group were killed on each of postaxotomy days 7, 14, and 28 (corresponding to days 67, 74, and 88 after bone marrow transplantation). The animals were perfused with normal saline to remove any intravascular circulating cells. Retinal DNA was extracted (Easy-DNA kit; Invitrogen, Carlsbad, CA), and the pellet was dissolved in 50 µL Tris EDTA and quantified. Similarly, DNA samples from the spleens and livers were obtained and quantified. Polymerase chain reaction was performed with the primers and 35 cycles as described earlier.

Resident Retinal Microglia in the Chimera

This experiment was designed to test whether total body irradiation and bone marrow transplantation may by themselves alter the number of retinal microglia. Sixty days after the construction of chimeras, three chimeric rats and three nonchimeric rats underwent right optic nerve axotomy with application of 4Di-10ASP, as described earlier. Fourteen days after the axotomy, the animals were killed, and the enucleated eyes were processed for flat preparation and mounted for confocal visualization. Five retinal fields adjacent to the optic disc were randomly selected, and the number of 4Di-10ASP-positive cells in 0.09 mm² was counted.

Macrophages in EAU Chimeric Rats

Two months after the chimeric construction, EAU was induced in four chimeric (Y→X) rats by immunizing with S-antigen peptide as described earlier. Two animals each were killed on postimmunization days 10 and 12. Histopathologic examination was performed on half of the retina and the whole anterior segment from each eye. DNA was extracted from the remaining retina (Easy-DNA kit; Invitrogen) and subjected to Y chromosome detection by PCR amplification, as described earlier.

Generation of TNFα and Peroxynitrite by Retinal Microglia in EAU

Twelve Lewis rats were axotomized and 4Di-10ASP was deposited on the cut stump of the optic nerve, as described earlier. At postaxotomy day 28, all rats were immunized with S-antigen peptide, also described earlier. Three animals were killed on each of days 6, 8, 9, and 10 after S-antigen injection. The enucleated globes were divided into two half portions, and each half was tested for either TNFα or peroxynitrite (ONOO⁻). For TNFα localization, 14-µm cryostat sections of the retina were fixed with 4% paraformaldehyde at room temperature for 10 minutes and then exposed to primary antibody and goat anti-rat TNFα (1:100, R&D Systems, Minneapolis, MN) and subsequently to a secondary antibody, rhodamine-conjugated anti-goat IgG (1:100; Vector Laboratories). The immunostained sections were examined by confocal microscope as described earlier; except, the 543-nm laser beam for the excitation of rhodamine fluorescent was switched off for visualization of 4Di-10ASP, and the 488-nm excitation beam was switched off for visualization of rhodamine, to eliminate any possible cross talk between the two fluorophores.

For detection of ONOO⁻, the retinas were exposed to dihydrorhodamine 123 (Molecular Probes) in medium supplemented with B27 (Gibco BRL). The dihydrorhodamine was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) to a concentration of 5 mM, and this solution was further diluted to a 50-µM working solution with Dulbecco’s modified Eagle’s medium. Retinas were incubated in working solution for 3 hours before 14-µm frozen sections were obtained. Visualization was performed with the confocal scanning microscope, as described earlier.

RESULTS

In Vivo Labeling of Retinal Microglia

At 3 days after axotomy, the flat preparation of the retina revealed the presence of fluorescent dye (4Di-10ASP) within the ganglion cells. These labeled cells were large, measuring approximately 30 µm. At postaxotomy day 14, a few such labeled ganglion cells were detected, but there were several smaller cells, measuring approximately 10 to 14 µm, which contained the dye. These smaller cells stained positive for OX42. The ganglion cells were negative for OX42 (Fig. 1). Several cells that were positive for both 4Di-10ASP and OX42 displayed dendritic extensions.

Migration of Retinal Microglia in EAU

In the nonimmunized rats killed on postaxotomy day 3, the retina revealed only labeled ganglion cells (Fig. 2A). At postaxotomy day 14, several cells were positive for both 4Di-10ASP and OX42 in the nerve fiber layer and the inner plexiform layer (Fig. 2B). On day 38, the ganglion cells disappeared, but there were numerous microglia (positive for both 4Di-10ASP and OX42). These microglia were noted in the nerve fiber and inner plexiform layers (Figs. 2C–E).

At days 7 and 8 after S-antigen peptide injection, the enucleated globes of the rats showed the presence of microglia (OX42- and 4Di-10ASP-positive) in the inner retina. None of these cells were seen in the outer nuclear and photoreceptor cell layers. On the ninth day after immunization with the S-antigen peptide, the enucleated globes showed several microglia (both OX42- and 4Di-10ASP-positive) in the outer nuclear and photoreceptor layers of the retina (outer retina) and only a small number of such microglia in the inner retina (Figs. 3A–C). There were no macrophages (OX42-positive, 4Di-10ASP-negative cells) in the inner or outer retina. Histologically, the retinal architecture was relatively intact (Fig. 3D). The microglial population increased in the outer retina on postimmunization day 10, and there was mild disruption of the retinal architecture. Moreover, no infiltration of macrophages (OX42-positive, 4Di-10ASP-negative cells) was detected. However, at postimmunization day 11, there was infiltration of both microglia and macrophages in the outer retina. There was clear histologic evidence of a disrupted retina associated with mononuclear cell infiltration. The number of macrophages (OX42-positive, 4Di-10ASP-negative cells) substantially increased on day 12 after immunization (Fig. 4). At this stage, there was a marked disruption of the retina and infiltration of mononuclear cells. Morphometric analysis revealed a progressive increase in the number of microglia in the outer retina on days 9 and 10 after immunization. Macrophage infiltration, in contrast, first appeared in the retina on day 11, and the cells became more numerous on day 12 (Fig 5).

Detection of Y Chromosome–Containing Cells by PCR

The resultant 104-bp fragment sequence matched with that of male Y-chromosome-specific 104-bp fragment (EMBL database; Accession number X89750). EMBL is provided in the public domain by the European Molecular Biology Laboratory, Heidelberg, Germany, and is available at http://www.embl-heidelberg.de/). The PCR amplification products elicited with the primers demonstrated the presence of 104-bp fragments in the male DNA, but not in female DNA. Using the male-female splenic DNA mixture, this PCR protocol detected 1 ng of male DNA in 1 µg of female DNA (0.1%) and could detect 0.01% male cells (1:10,000) in the mixture of male and female cells.

Construction of Chimeric Rats and Determination of the Stability of the Retinal Microglia Compartment

PCR analysis of DNA extracted from the spleens of the three chimeric animals 2 months after (Y→X) bone marrow trans-
plantation revealed the presence of a Y-chromosome-specific 104-bp fragment, indicating the successful construction of chimeric animals. Neither the retina of the right axotomized eyes nor the retina of the left nonaxotomized eyes of the chimeric animals revealed the presence of Y chromosome, irrespective of whether the rats were killed on postaxotomy day 7, 14, or 28. These results indicate that the retinal microglial compartment is stable and devoid of any repopulation by peripheral blood-derived cells, even 88 days after bone marrow transplantation. At this stage, DNA obtained from the spleen and lungs of these animals showed the 104-bp amplified product.

### Resident Retinal Microglia in the Chimera

In the control rat retinas, there were $46.7 \pm 9.4$ microglia/0.09 mm², whereas in the chimeric retinas, there were $53.2 \pm 13.0$ microglia/0.09 mm². Although there appeared to be a trend toward an increased number of microglia in the chimeric animal retinas, the difference between the chimeric and control animals was not significant ($P = 0.07$; Fig. 6).

### Macrophages in EAU Chimeric Rats

In the chimeric (Y→X) animals injected with the S-antigen peptide, one of the four eyes showed the PCR amplification product of the Y chromosome on day 10. The same eye also exhibited histologic evidence of inflammation, including infiltration of polymorphonuclear neutrophils (PMNs) and mononuclear cells and disrupted retinal architecture. The other three 104-bp negative eyes showed no such evidence of inflammatory cell infiltration, and the retinal architecture was intact. In contrast, on day 12, three of the four eyes were positive for Y chromosome (Fig. 7), and there was histologic evidence of retinitis, characterized by infiltration of monocytic cells and disrupted retinal architecture.
Generation of TNFα and ONOO⁻ by the Migrated Retinal Microglia in EAU

On the sequential evaluation to detect TNFα in the retina after injection of S-antigen peptide, rats killed on days 6 and 8 were all negative for TNFα staining, but the rats killed on days 9 and 10 showed positive staining for TNFα within the 4Di-10ASP-positive cells (microglia). The dual-labeled (4Di-10ASP-positive and TNFα-rhodamine-positive) cells were localized predominantly in the photoreceptor layer, and the TNFα-rhodamine-positive cells matched the 4Di-10ASP-labeled microglia that migrated to the outer retina (Fig. 8). There was also a small population of dual-labeled cells in the inner retina.

In the sequential study covering days 6, 8, 9, and 10 after S-antigen peptide injection, the oxidation of dihydrorhodamine to fluorescent rhodamine was not visible at days 6 and 8. Moreover, there were no 4Di-10ASP-positive cells in the outer retina. However, the retinal sections of the animals killed on days 9 and 10 after S-antigen peptide injection showed 4Di-10ASP-labeled cells at the photoreceptor layer. These cells also revealed the presence of rhodamine fluorescence. These dual-positive cells were more numerous on day 9.
DISCUSSION

Optic nerve axotomy specifically labels the microglia through phagocytosis of 4Di-10ASP containing degenerating ganglion cells (Figs. 1, 2). The 4Di-10ASP dye was initially seen in the ganglion cells and subsequently only in OX42-positive cells. In this labeling process, no astrocytes, Müller cells, or other neuronal cells were labeled with 4Di-10ASP, indicating that the dye does not simply leak from the degraded ganglion cells, but is taken up and internalized by the OX42-positive cells in a process specific only to phagocytosis. In this study, we found that although only 30% to 50% of the degenerating ganglion cells were labeled by 4Di-10ASP delivered from the optic nerve, these ganglion cells are sufficient to label the entire population of retinal microglia, as indicated by the microglial staining with the dye (Fig. 2C) and with OX42 (Fig. 2D). All the 4Di-10ASP–labeled microglia are also labeled with OX42 (Fig 2E). EAU was not induced in these animals, and therefore, macrophages are absent. Thanos and Thanos et al. arrived at the same conclusion by staining the microglia with OX42. Therefore, it is safe to conclude that almost all retinal microglia were labeled, and thus we were able to trace the migratory behavior of the retinal microglia.

The PCR (for the Y chromosome) of the retinas from axotomized chimeric animals showed the absence of the Y-chromosome–amplified product, indicating that the 4Di-10ASP–positive and OX42-positive cells are indeed microglia. Thus, this complex experimental approach could discern microglia from circulation-derived macrophages (Y-chromosome–possessing cells). Moreover, EAU induction in such animals showed that the macrophages (OX42-positive, 4Di-10ASP-negative cells) appeared in the retina when PCR for the Y chromosome became positive (Figs. 3, 4, 7), indicating that the macrophages did not phagocytose the dye-containing cellular debris. This allowed us to use a morphologic approach to

**FIGURE 3.** Microglial migration to the photoreceptor cell layer on day 9 after immunization. In the early phase of EAU, postimmunization day 9, the 4Di-10ASP–positive microglia migrated to the photoreceptors (A). These cells were also positive for OX42 (B). The superimposed image of the dye and OX42 staining indicates the colocalization (C, yellow). (D) Well preserved retina on day 9 (toluidine blue). I, inner retina; O, outer retina. Magnification, ×300.
positively distinguish the microglia from macrophages. The microglia were positive for both 4Di-10AP and OX42, and the macrophages were positive for OX42, but negative for 4Di-10ASP.

In the present study, we took advantage of the initial period at days 28 to 30 postaxotomy, when microglia became stabilized after phagocytosis of the ganglion cells. After the induction of EAU in the axotomized rats, the retinal microglia (dual stain-positive for OX42 and 4Di-10ASP) migrated to the outer retina–photoreceptor cell layer at the early phase of EAU (days 9 and 10 after immunization). When the disease reached the amplification phase (days 11 and 12 after immunization), several of the OX42-positive cells were negative for 4Di-10ASP (macrophages), whereas others that were positive for both 4Di-10ASP and OX42 (microglia) were localized to the photoreceptor layer. The PCR of the retina for the Y chromosome became positive at this amplification phase. These findings clearly show that microglia migrate to the outer retina during the initial phase of uveitis, and macrophages subsequently appear during the amplification phase. These results confirm that the cells that initially appear at the site of the target retinal antigen are microglia and suggest that they may play a role in the recruitment of bone marrow–derived cells at the site of the target antigen. Moreover, we found that the migrated microglia release two proinflammatory mediators, TNFα and ONOO⁻ (Figs. 8, 9), in the photoreceptor cell layer before the arrival of macrophages. The release of these mediators suggests that the microglia participate effectively in the initiation of the inflammatory effector process that leads to photoreceptor degeneration.

In our EAU study, the microglia migrated early to the photoreceptor layer, before the apparent degeneration of photoreceptors. Therefore, it appears that they are not recruited by the degenerated outer segment debris. Although the putative chemotactic signal transmitted to the retinal microglia remains elusive, the products of membrane lipid peroxidation were

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933224/)
suggested as the mediator for migratory events. For example, 4-hydroxynonenal, an aldehydic product of lipid peroxidation, was able to activate microglial cells through an interaction with microglial scavenger receptors. We have demonstrated that in EAU, docosahexaenoic acid (22:6), a major membrane fatty acid in photoreceptors, is peroxidized to form hydroperoxide (22:6 HP). Further, we have demonstrated that 22:6HP is chemotactic to phagocytic cells and microglia. Therefore, it is conceivable that such hydroperoxides play a role in the migration of microglia in EAU and related retinal inflammations. In consideration of the recent study in which the S-Ag-specific T-cells were shown to be present in the retina within a few hours after adoptive transfer, we must also acknowledge the possibility that the T-cell-mediated early inflammatory products can also serve as the chemoattractant(s) for the migration of these microglia.

The retinal microglia have been shown to be highly migratory, especially in response to retinal insults such as optic nerve transection, focal laser lesions, photoreceptor degeneration, and IL-1-induced inflammation. More recently, exposure to light has been found to induce migration of mouse retinal microglia into the subretinal space, with the unjured aging photoreceptors acting as the chemoattractant that recruits the microglia from the outer nuclear layer to the subretinal space. However, the exact mechanism underlying the activation and subsequent migration of microglia is still largely unknown.

Although microglia have low mitotic activity, for chimeric animals to be useful in delineating retinal microglia from the blood-derived monocytes, the stability of the microglial compartment must be maintained during the entire period of the experiment and beyond. It is also important that any subsequent experimental intervention, such as transection of the optic nerve, not change this stability. On postaxotomy day 14 in the chimeric rats (day 74 after bone marrow transplantation), we found that the retinal microglial cell counts in the chimeric and nonchimeric rats were not the same. However, the difference between the two groups was small and not significant (P = 0.07). Using PCR detection, repopulation engraftment was found to be rapid in hematopoietic tissues such as the spleen and, to a lesser extent, the lung. This repopulation was absent in the retina and the brain parenchyma as much as 30 weeks after the transplantation. These results are consistent with recently a published kinetic study of engraftment in the mouse after bone marrow transplantation, in which the difference between the two groups was small and not significant (P = 0.07). Using PCR detection, repopulation engraftment was found to be rapid in hematopoietic tissues such as the spleen and, to a lesser extent, the lung. This repopulation was absent in the retina and the brain parenchyma as much as 30 weeks after the transplantation. These results are consistent with recently a published kinetic study of engraftment in the mouse after bone marrow transplantation, in which the difference between the two groups was small and not significant (P = 0.07). Using PCR detection, repopulation engraftment was found to be rapid in hematopoietic tissues such as the spleen and, to a lesser extent, the lung. This repopulation was absent in the retina and the brain parenchyma as much as 30 weeks after the transplantation. These results are consistent with recently a published kinetic study of engraftment in the mouse after bone marrow transplantation, in which the difference between the two groups was small and not significant (P = 0.07).
we transplanted marrow from male Lewis rats to female Lewis rats. When Lewis rats and (DAxLewis) F1 hybrids are used as bone marrow donors, the meningeal and perivascular monocytes are slowly replaced by hematogenous cells under normal conditions, whereas resident microglia represent a stable cell pool that is only exceptionally replaced by hematogenous cells in adult animals. These results contrast with a higher rate of repopulation encountered in xenogenic transplantation, in which graft-versus-host disease is evident. Our experimental approach clearly shows that the retinal microglial compartment is a stable one, even at 30 weeks after transplantation, indicating that there is a stable microglial compartment at the time of development of EAU without engraftment of blood-derived monocytes in the retina.

In vitro studies on microglia show that these cells produce superoxide, nitric oxide, and TNF. The relationship of these cytotoxic agents to neuronal death has been verified by an experiment in which inhibition of microglial invasion into the photoreceptor cell layer promotes the survival of photoreceptor cells in the dystrophic retina. In our EAU animals, the microglia were recruited to the photoreceptor layer very early in the disease state, particularly before infiltration of hematogenous macrophages and other phagocytes. Moreover, our histochemical observations showed that microglia that are localized at the photoreceptors generate TNF and ONOO\(^-\) (Fig. 8) and ONOO\(^-\) (Fig. 9). ONOO\(^-\) is the most potent biological oxidant capable of oxidizing cellular components. In CNS inflammation, microglia continue to express TNF as part of the maintenance and amplification of the inflammatory cascade. In vitro data suggest that TNF can cause the release of other cytokines, such as IL-1\(\beta\) and IFN\(\gamma\), from reactive astrocytes. Unlike the production of oxidants, production of TNF is not associated with phagocytosis. Therefore, the production of TNF by the migrated microglia before the arrival of blood-born phagocytes in the EAU indicates that microglia have a proinflammatory and pathogenic role, rather than the mere phagocytic function of eliminating the damaged photoreceptors.

In summary, the precise in vivo identification of activated retinal microglia and their migration into the photoreceptor layer before the infiltration of hematogenous cells in the initial
phase of uveoretinitis reveals a novel pathogenic mechanism by which the retinal inflammation may begin in autoimmune uveoretinitis and by which subsequent retinal degeneration may take place in the inflammatory process. These findings further expand current belief about the role of microglia, in which only their scavenger role, rather than their pathogenic role, in retinal diseases has been emphasized. Because retinal damage in uveitis is caused by the amplification process, a better understanding of the initial inflammatory process may lead to the development of novel, locally acting, anti-inflammatory agents directed to the microglia. Such a specific treatment would be ideal, in that it would avoid the present day systemic treatment with its associated complications.

References


