Complement Component 1Q (C1Q) Upregulation in Retina of Murine, Primate, and Human Glaucomatous Eyes

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Purpose. Complement has been implicated in the pathogenesis of neurodegenerative diseases. The purpose of this study was to investigate whether complement activation is part of the pathogenesis of retinal ganglion cell (RGC) loss in glaucoma.

Methods. mRNA and protein was extracted from the retina and brain of DBA/2 and C57/BL6 mice and subjected to RT-PCR and immunoblot analysis, respectively. In addition, eyes from the same mouse strains were subjected to immunohistochemistry with antibodies specific to complement component 1q (C1q). Eyes from monkeys with unilateral experimental glaucoma were also subjected to immunohistochemical analysis, as were eyes from human subjects with or without glaucoma.

Results. C1q mRNA and C1q protein were found to be upregulated in the retina of glaucomatous DBA/2 mice. Upregulation of C1q preceded the time of extensive RGC death and increased with increasing age to 15 months in the retina, but not in the brain. No age-related C1q upregulation was detected in the reference mouse strain (C57BL/6), which develops significant nonglaucomatous RGC loss toward the end of the same time frame. C1q upregulation was also detected in laser-induced glaucomatous monkey eyes and in some (but not all) eyes of patients with glaucoma. C1q upregulation was localized to the Müller cells within the retina and in the area of the inner limiting membrane.

Conclusions. Complement expression is upregulated in the retina of two commonly used glaucoma models (in the DBA/2 mouse and the monkey) and in some human glaucomatous eyes. The timing of this upregulation suggests that complement activation plays a significant role in the pathogenesis of glaucoma. (*Invest Ophthalmol Vis Sci.* 2006;47:1024–1029) DOI:10.1167/iovs.05-0830

The complement is an ancient phylogenetic defense mechanism that, in mammals, comprises several soluble proteins produced by many cell types. It has diverse functions that include antigen opsonization in preparation for phagocytosis, destruction of invading pathogens, facilitation of apoptotic cell removal, and inflammatory response enhancement.[1]

C1q is part of the activation arm of complement. It is the first element in the classic complement activation pathway that starts on binding of C1q to the antigen, either directly or through an antibody–antigen complex. C1q then interacts with and activates several proteases (C1r, C1s, C2-C4) that amplify the original response and initiate opsonization, anaphylactic reactions that attract phagocytes, and that finally directly attack the cell membrane through the membrane attack complex (MAC). In addition, C1q appears to have other potential functions through cell-specific receptors.[2,3]

Tissues that are known to express C1q include the heart[4] and brain.[5] In the brain, C1q can be induced by kainic acid and decortication in rats.[6] C1q also appears to be involved in the pathogenesis of scrapie, as disease development is delayed in mice without C1q.[7,8] Increased neuronal C1q expression occurs in Alzheimer's disease (AD), and thus the role of C1q in the pathogenesis of AD has been the subject of considerable investigation, as reported in recent reviews.[9,10] Damaged neuronal processes show evidence of MAC activation,[11] with concurrent increase in the expression of C1q[12] that is not paralleled by increases in the protective C1 inhibitor,[12] indicating a complement-mediated autotoxic reaction. It has been proposed that microglia that carry receptors to C1q could exacerbate the inflammatory response in the AD brain.[13] In contrast, C1q can bind to amyloid plaques in AD, reducing amyloid β-peptide (Aβ) uptake and thus leading to extracellular accumulation of Aβ.[14]

Recent reports[15,16] have suggested that components of the complement activation arm including C1q are upregulated in the retina of animal models of glaucoma. This suggests that C1q may be associated with glaucomatous pathology in the retina and that this condition has features in common with other neurodegenerations. To characterize further the temporal and spatial distribution of this upregulation in the retina in relationship to glaucomatous RGC damage, we investigated C1q expression in DBA/2 mice, which undergo spontaneous development of a form of angle-closure glaucoma; in monkey eyes with experimental glaucoma; and in human eyes with glaucoma.

Methods

Animals and Tissues

DBA/2J and C57BL/6 mice of ages ranging from 3 to 18 months were studied. Mice were kept in a 12-hour light–dark cycle and fed ad libitum. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care Committee. Experimentation on human specimens was approved by the Institutional Review Board.

Mice were anesthetized with a mixture of ketamine, xylazine, and acepromazine (10.8, 1.2, and 54 mg/kg) before death. Blood was aspirated from the left ventricle. Mice were then perfused with a

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TABLE 1. Characteristics of Human Specimens Used in the Clq Immunohistochemisty Experiments

	Glaucomatous Eyes	Control Eyes
Number	11	11
Age, y (mean ± SD)	64.8 ± 21.9 years	64.3 ± 19.3 years
Sex*		
Male	1	8
Female	10	3
Source of specimens		
Autopsy	3	11
Enucleation	8	0
Type of glaucoma		
Chronic angle closure	4	
Aphakic	1	
Juvenile	1	
Phacomorphic	1	
Congenital	1	
Unspecified	3	

Most glaucomatous eyes were obtained following enucleation for end-stage painful glaucoma. In contrast, all of the control eyes were obtained at autopsy.

* $P < 0.05$.

solution containing heparin (1,000 U/mL) in normal saline to remove the remaining blood from the vascular tree, and the eyes and brain were immediately removed. The retina was isolated and stored (RNAlater; Ambion, Austin, TX) at −20°C, as was the brain. Blood serum collected was also stored at −80°C after centrifugation at 1000 rpm for 5 minutes in the presence of a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN).

Mice used for immunohistochemistry were initially perfused with the same solution, to remove blood from the vascular tree, and then with 4% ice-cold paraformaldehyde in phosphate-buffered saline (PBS). Eyes were enucleated and further fixed in the same fixative for 24 to 72 hours before further processing.

Enucleated eyes from two adult female cynomolgus monkeys with unilateral experimental glaucoma for more than 5 years were studied. In these two animals, glaucoma had been induced by repeated diode laser photocoagulation of the midtrabecular meshwork in the right eye. Both experimental eyes had high IOP (range, 25–40 mm Hg) and apparent optic nerve damage. The left (untreated) eye of each animal was used as the control. The animals had in the past participated in other experiments to evaluate ocular hypotensive agents, but had not received any topical or systemic medications for at least 4 weeks before death. The eyes were enucleated at the time of euthanasia and preserved in 10% formalin for at least 3 weeks before processing.

Archival material from patients having undergone enucleation for a variety of reasons was also studied. Eyes of patients without significant ocular history that were obtained after death served as the control. Specimens were included in the experimental group if the clinical history indicated the presence of glaucoma (any type) or if the histologic examination revealed the presence of typical glaucomatous optic neuropathy. Specimens were excluded if the clinical history or histologic analysis indicated the presence of vitreous hemorrhage or active inflammation. Specimens had been fixed by immersion in 10% buffered formalin at the time of enucleation and were embedded in paraffin. Clinical data of the human eye samples used are summarized in Table 1. Because information on the specimens was obtained through pathology records (and not through the patients' records), information on treatment and IOP control is not complete. All eyes in this category showed histologic evidence of advanced glaucomatous neuropathy.

Reverse Transcription–Polymerase Chain Reaction

Total RNA and protein were extracted (TRIzol protocol; Invitrogen-Gibco, Grand Island, NY). Tissue was homogenized in the extraction reagent, mixed with chloroform, and centrifuged, and then the aqueous and the nonaqueous phases were separated. The nonaqueous phase was subjected to dialysis to recover the proteins (described later), whereas the aqueous phase containing the RNA was further purified (RNeasy Micro Kit; Qiagen, Valencia, CA). The amount of RNA isolated was quantitated spectrophotometrically and the concentration adjusted for subsequent reverse transcription. Reverse transcription using random primers was performed (Superscript First Strand Synthesis System for RT-PCR protocol; Invitrogen, Carlsbad, CA).

Real-time PCR was performed using primers designed based on the sequence of mouse C1qb mRNA (GenBank NM_009777; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The anticipated product size was 193 bp. Primer sequences were: CCAACGCGAACGAGAACTAT (forward) and GTGGTCACCTGGAAGGTGTT (reverse). To quantitate accurately the amount of C1q in the various samples and account for variations that may be introduced by variable efficiency of reverse transcription, the product of a housekeeping gene was also amplified from the same cDNA samples in separate reactions. The gene *rps11* (*Mus musculus* similar to 40S ribosomal protein S11, GenBank XM_193290) encoding for a ribosomal protein, was used for this purpose. Primer sequences used for *rps11* amplification were CGTGACGAAGATGAAGATGC (forward) and GCACATTGAATCGCACAGTC (reverse). The PCR amplification (SYBR Green; Applied Biosystems, Inc. [ABI], Foster City, CA) was performed at three cDNA dilutions (1, 10, and 100 ng) for each run, with each dilution in triplicate. Reactions were run at least twice for each mouse strain and age group, tissue, and cDNA level. Reactions were run on a sequence-detection system (Prism 7900HT; ABI), and results were analyzed (SDS 2.1 software; ABI). Normalized relative concentrations of C1q mRNA in the various age groups were compared with analysis of variance (ANOVA) and post hoc Fisher least-significant difference (LSD) testing.

Immunoblot Analysis

The nonaqueous phase from the extraction (TRIzol; Invitrogen-Gibco) was dialyzed through a 3500-Da membrane (Millipore, Bedford, MA) at 4°C for 48 hours in 0.1% SDS and then centrifuged at 10,000*g* for 10 minutes. The supernatant was stored at −80°C until used. After resuspension in a buffer containing 25 mM Tris (pH 1.7), 1.6% SDS, 8% glycerol, and 0.7 M β-mercaptoethanol (βME) samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes (HybondECL; GE Healthcare, Piscataway, NJ). Nonspecific binding was blocked with blocking buffer (SuperBlock; Pierce, Rockford, IL) in PBS. Membranes were then incubated with the primary antibodies (anti-C1q, A301 at 1:4000; Quidel, San Diego, CA) and anti-actin (sc1616 at 1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with a horseradish peroxidase (HRP) chemiluminescence system (ECL; GE Healthcare). Luminescence was measured with an automated imager (model 440CF, digital imaging station; Eastman Kodak, Rochester, NY). Relative optical density of each band was measured using the Image Tool software package (University of Texas Health Science Center, San Antonio). The amount of C1q in each lane was normalized in relationship to the amount of the housekeeping protein (actin) in the same lane. Normalized concentration graphs were created (a minimum of five membranes analyzed per point). Normalized concentrations were compared by using ANOVA with post hoc Fisher LSD testing. Immunoblots of both individual animal tissues as well as tissues pooled from several animals (minimum pool from five eyes per lane) were performed to account for individual eye variability in the amount of RGC loss in the DBA/2 retina.[17]

Positive controls included purified human C1q protein (Quidel) and mouse blood and rat testis lysate (BD Biosciences, Franklin Lakes, NJ) for C1q and actin antibodies, respectively. Specificity of the C1q antibody for the intact protein was confirmed by elimination of staining with complete disulfide bond reduction of the molecule[18,19](data not shown) as well as lack of staining on omission of primary antibody.

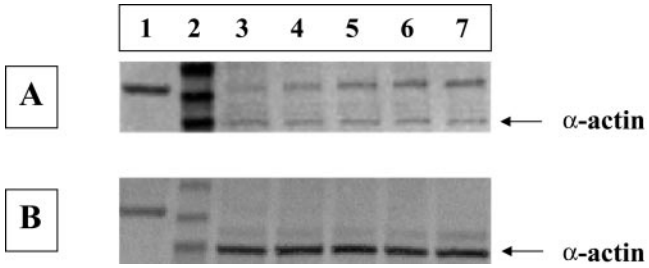

FIGURE 1. C1q and actin immunoblot analysis of DBA/2 retina (**A**) and brain (**B**). DBA/2 tissue was pooled from at least five individual eyes for (**A**) and at least five individual animals for (**B**). C1q immunoreactivity in retina markedly increased with age, whereas it remained undetectable in the brain of similarly aged animals. *Lane 1*: mouse blood serum (C1q positive control); *lane 2*: molecular weight marker; *lane 3*: 3-month-old animals; *lane 4*: 6-month-old animals; *lane 5*: 9-month-old animals; *lane 6*: 12-month-old animals; and *lane 7*: 15-month-old animals.

Immunohistochemistry

Sections of the tissues (4–5 μm) were collected on positively charged slides (ESCO, Superfrost Plus Microscope Slides; Erie Scientific, Portsmouth, NH) and dried in an oven for 1 hour at 60°C. Sections were then deparaffinized in xylene and rehydrated in graded alcohols. The tissues were digested with proteinase K (0.03% in distilled water for 20 to 40 minutes). Testing of the human specimens under various digestion lengths was necessary because of the variable conditions of time to fixation, amount of fixation, and processing of this archival material. Sections were then rinsed three times for 3 minutes with PBS and incubated with the primary antibodies (anti-C1q 1:200; Quidel; anti-glutamine synthase 1:50, BD-Transduction Laboratories, Lexington, KY) for 2 hours at room temperature in a solution containing 1% BSA in PBS. They were then rinsed three times with PBS and treated with the appropriate Alexa-conjugated secondary antibody (1:400; Molecular Probes, Eugene, OR) for 20 minutes at room temperature. After the sections were rinsed, they were treated with 4′,6′-diamino-2-phenylindole (DAPI; 5 μg/mL) and washed, and the slides were coverslipped and observed with a microscope (Axioscope; Carl Zeiss Meditec, Inc., Dublin, CA), with the appropriate excitation wavelengths and filters. Antibody specificity for the C1q antibody was verified by immunoblot analysis (data not shown), elimination of staining with the addition of excess antigen, and the absence of C1q staining in sections of $C1q^{-/-}$ animals[20] (kindly provided by Marina Botto, Rheumatology Section, Faculty of Medicine, Imperial College, London, UK). Negative controls were incubated without primary antibodies.

For human specimens in particular, staining was considered positive if it was present in at least part of the retina under any of the two digestion protocols (20 or 40 minutes).

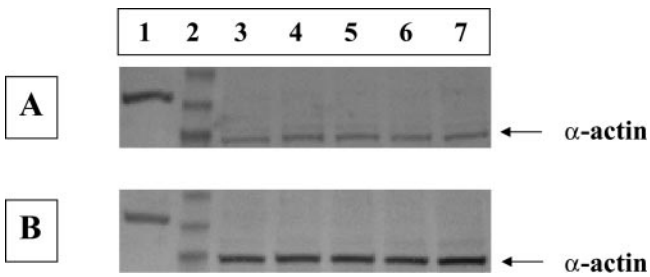

FIGURE 2. C1q and actin immunoblot analysis of C57BL/6 retina (**A**) and brain (**B**). C57BL/6 tissue was pooled from at least five individual eyes for (**A**) and at least five individual animals for (**B**). C1q immunoreactivity in the retina does not increase with age, and it remains undetectable in the brain of similarly aged animals. The contents of the lanes are as in Figure 1.

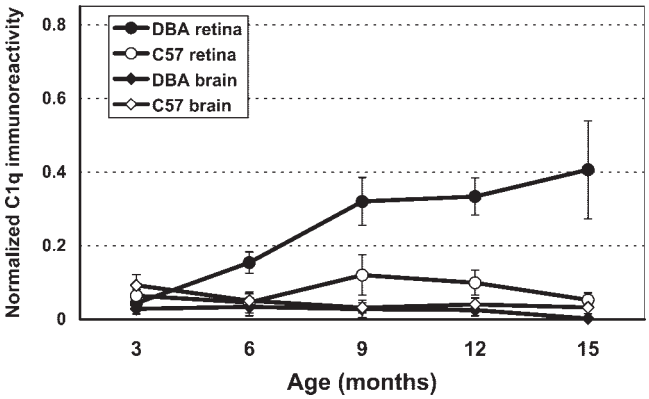

FIGURE 3. C1q immunoreactivity in retina and brain of 3-, 6-, 9-, 12-, and 15-month-old DBA/2 and C57BL/6 mice. C1q staining was normalized to the staining of actin to account for potential differences in the amount of protein loaded in each lane. C1q staining significantly increased in the retina of the DBA/2 mice starting at 6 months of age. Error bars: SEM from five different Western blot membranes. Each sample contains protein from a pool of at least five individual eyes from the corresponding age group.

RESULTS

Immunoblot analysis of retinas from DBA/2 mice of various ages (protein pooled from at least five eyes per lane) revealed increasing amounts of C1q with age, starting at approximately 6 months (Fig. 1A). In contrast, such an increase in C1q expression did not occur in the brain of the DBA/2 animals (Fig. 1B) or in the retina and brain of the C57BL/6 mice (Fig. 2). The time course of changes in the amounts of C1q in the retina and brain of DBA/2 animals and the control C57BL/6 mice is shown in Figure 3. C1q immunoreactivity was constant in DBA/2 brain and C57BL/6 retina and brain ($P = 0.77$, 0.19 and 0.21 respectively; ANOVA) whereas it increased in the DBA/2 retina ($P < 0.002$, ANOVA). This increase starts after the 6-month point ($P < 0.05$ Fisher LSD post hoc test).

A significant variability in C1q amounts was found in eyes of older DBA/2 mice, as can be seen in immunoblots from individual retinas of 15-month-old mice (Fig. 4).

To confirm that the C1q detected on immunoblot analysis was locally synthesized in the retina (rather than transported there through the bloodstream) real-time PCR was performed with retinal tissue from pooled eyes. As can be seen in Figure 5, significant upregulation of C1q expression started after 6 months of age and progressively increased to 15 months of age ($P < 0.0002$, ANOVA, Fisher post hoc LSD test). This increase was again in contrast to the small changes in the expression of C1q in same-aged C57BL/6 retinas (Fig. 5).

Retinal immunohistochemistry revealed the presence of C1q in the inner retina of aged DBA/2 mice, specifically on Müller cells and the inner limiting membrane (Fig. 6). C1q

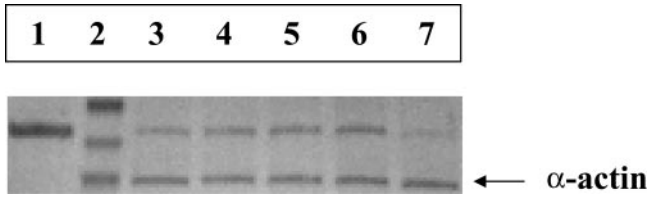

FIGURE 4. Retinal C1q and actin immunoblot analysis of individual eyes from 15-month-old DBA/2 mice. Immunoreactivity varied significantly between individual eyes. *Lanes 1*: mouse blood serum (C1q positive control); *lanes 2*: molecular weight marker, *lanes 3* to *7*: Retinal protein from five individual eyes.

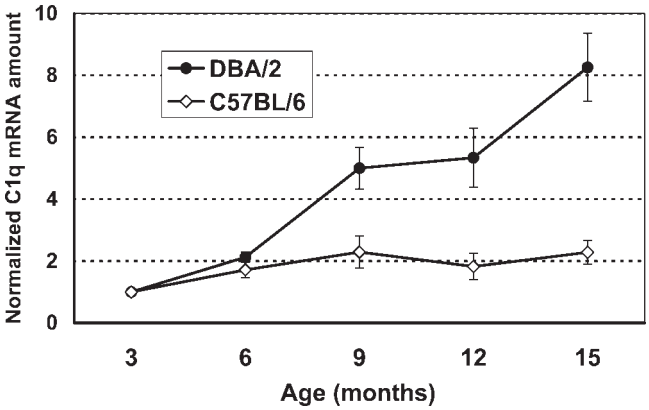

FIGURE 5. C1q mRNA in retina of 3-, 6-, 9-, 12-, and 15-month-old DBA/2 and C57BL/6 mice. C1q mRNA was normalized to the amount of the housekeeping ribosomal gene *rps11*, and the relative amount of mRNA at 3 months was set to 1. C1q mRNA significantly increased in the retina of the DBA/2 mice starting at 6 months of age. Error bars: SEM from three different RT-PCR experiments. Each sample contains RNA from a pool of at least five individual eyes from the corresponding age group.

immunoreactivity mostly colocalized with that of glutamine synthase, a marker for Müller cells (Figs. 6A, 6D). Young, prepathologic, 3-month-old DBA/2 mice showed only minimal C1q staining of Müller cells and no staining of the inner limiting membrane (Fig. 6B), whereas aged DBA/2 mice showed a dramatic increase of staining in these areas (Fig. 6E).

To determine the relevance of the findings to human disease, we compared immunohistochemical staining in primate eyes, with and without experimental glaucoma, and in glaucomatous human eyes in archival material. In long-standing glaucomatous monkey eyes C1q immunostaining was significantly increased in Müller cells (Fig. 7B), identified by GS staining (Figs. 7A, 7D), when compared with staining in the contralateral control eyes (Fig. 7E).

Clinical data on the human specimens stained are presented in Table 1. Staining for C1q was variable in the glaucomatous human eyes, with some of the glaucomatous eyes showing some staining of the inner limiting membrane. An example of such staining is shown in Figure 8B. Again, Müller cells are identified by GS immunostaining (Fig. 8A). Some of the control (nonglaucomatous) eyes also showed some C1q immunostaining in the area of the inner limiting membrane.

DISCUSSION

C1q is a large molecule made up of 18 polypeptide chains (6 of A, 6 of B, and 6 of C) held together by a combination of covalent (disulfide) and noncovalent bonds.[21–23] When assembled, the C1q full molecule has two distinct regions: a globular head and a long tail. Most of the antibodies raised against C1q are directed toward its globular head and recognize epitopes specific to the secondary structure of the assembled chains and are nonreactive to the amino acid sequence of each chain. The tightly bound individual polypeptide chains are difficult to separate from this complex, which requires long incubations under reducing conditions and high urea concentrations. Several proteins have globular domains similar to C1q. Therefore, we confirmed the specificity of the antibody used in this study by using purified human C1q and mouse serum, which has large amounts of C1q.

The expression of C1q in the retina has not been investigated. C1q expression has been reported to be upregulated in primate glaucoma by microarray analysis, but this finding[15] has not yet been confirmed at the protein expression level. In addition, expression of C1q and other components of the complement activation arm have been reported to be upregulated in the retina of ocular hypertensive rats.[16] Our results indicate an increase in the amounts of C1q in the retina of DBA/2 animals, in which glaucoma develops as they age, compared with young animals of the same strain.

The increase in C1q expression in the retina of DBA/2 mice started at approximately 6 months of age, was significant at approximately 9 months, and continued to 15 months. This increase in C1q expression parallels the time course of RGC loss but precedes it by at least 1 month in the DBA/2J animals (Danias J, unpublished data). The absence of such an upregulation in the brain of the same animals at the same time points suggests that it is specific for the retina. In addition, the C1q increase did not appear to be directly related to aging, as C57 mice (a reference nonpathologic strain) did not show any changes in immunostaining, even at advanced ages.

The initial increase in C1q immunostaining appears to coincide with the increase in IOP in these animals.[17,24] However, in contrast to the IOP, which returns to baseline after 12 months of age, C1q expression continues to increase up to at least 15 months.

DBA/2 mice have certain immunologic abnormalities that cause low-grade anterior uveitis between 4 and 7 months of age.[25] Given the temporal characteristics of C1q upregulation, it is unlikely that it is a direct consequence of this anterior chamber inflammation. It also does not appear to be part of a generalized neuroinflammatory phenomenon as other central nervous system (CNS) tissues do not show this upregulation.

Taken together, our results indicate that upregulation in C1q expression may be triggered by a stress caused by increased IOP on the retina of DBA/2 mice, but that its continuation may be related to the processes associated with RGC death. It is significant to note that C1q staining in the retina appeared to increase dramatically at about the time that significant RGC death begins in the glaucomatous DBA/2 mouse—10 to 12 months of age. In addition, the presence of significant variability of C1q amounts in DBA/2 retinas of 15-month-old animals paralleled the variability in the RGC loss seen at this age.[17]

The presence of increased immunostaining in the primate glaucomatous retina further supports the notion that C1q upregulation detected in murine glaucoma is relevant and may play a role in human disease. Human specimens from some patients with long-standing severe glaucoma also confirm the involvement of C1q in the pathophysiology of the disease; however, the results from experiments on archival material must be interpreted cautiously and further confirmed with experiments performed with more standardized processing. In addition, the specimens used for this study may not be representative of the full range of the disease. Most of the glaucomatous specimens came from women with end-stage glaucoma and were obtained at enucleation, whereas control specimens came mostly from men and were obtained at autopsy. This may account for the lack of cosegregation of C1q staining with the presence of glaucoma.

Localization of C1q staining by immunohistochemistry in both the mouse and primate eyes with glaucoma is intriguing. Expression of C1q in Müller cells (the glial cells that support and modulate the RGC environment) and possibly the retinal astrocytes that line the internal limiting membrane may be an adaptive mechanism used to remove apoptotic RGCs. Such a mechanism has been proposed for C1q expressing CNS neurons.[3] An alternative explanation could be that retinal glial cell dysfunction caused by high IOP or other unknown factors leads to C1q upregulation. The locally present C1q then binds to the RGCs causing cell death.

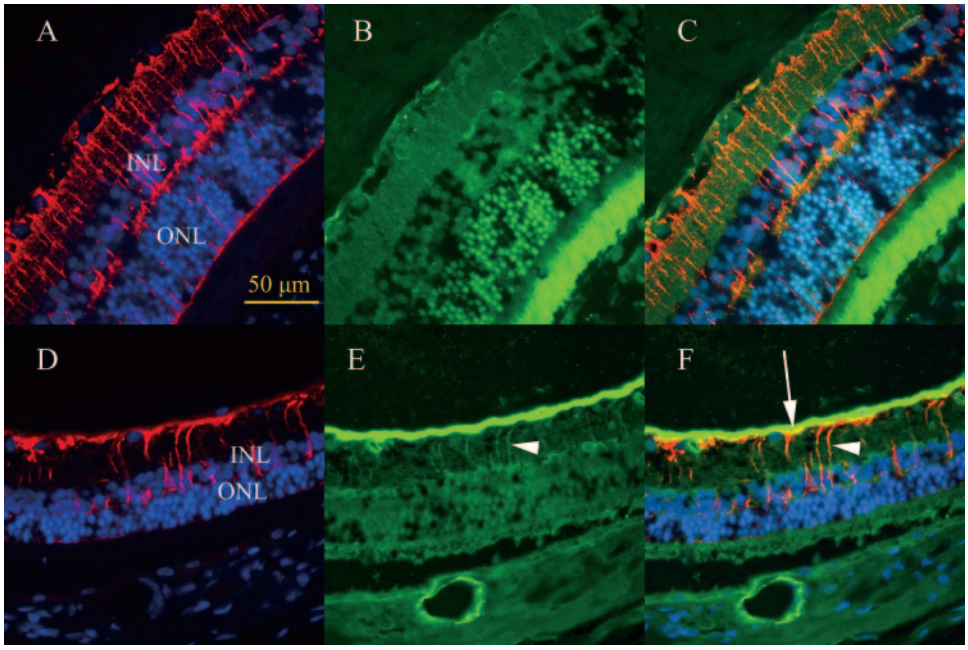

FIGURE 6. C1q (*green*, **B**, **E**) and glutamine synthase (*red*, **A**, **D**) immunostaining in sections from (**A–C**) 3- and (**D–F**) 20-month-old DBA/2 mice. (**C**, **F**) Merged images. *Blue*: DAPI-stained nuclei. Labeling of the inner limiting membrane was intense in the 20-month-old retina and was only partly caused by staining of the Müller end feet (*long arrow*). The processes of the Müller cells were also visible (*short arrow*). Very weak staining of Müller cell processes was also present in the 3-month-old retina. ONL, outer nuclear layer; INL, inner nuclear layer.

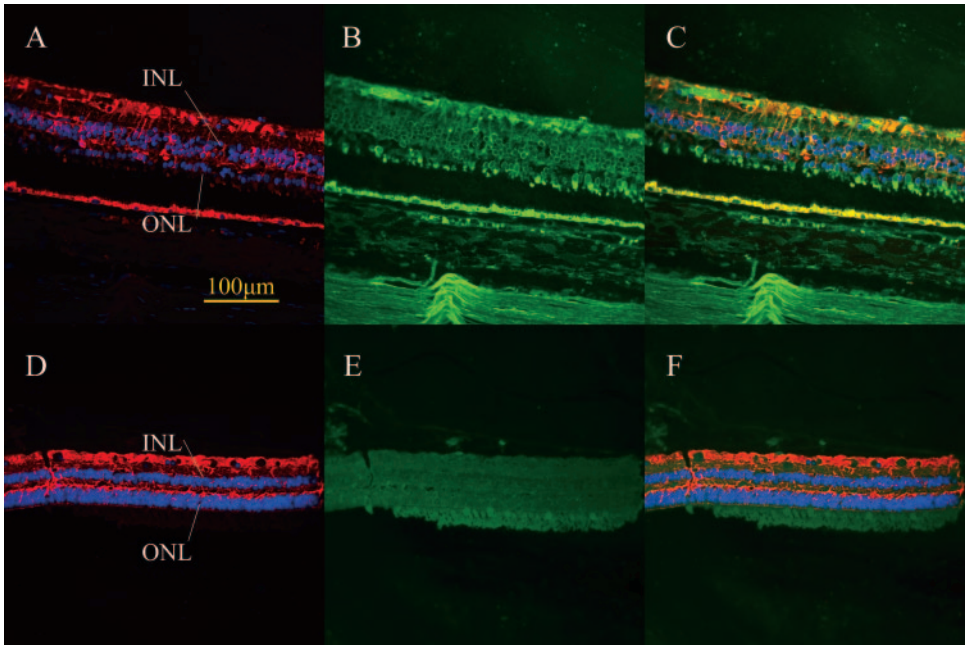

FIGURE 7. C1q (*green*; **B**, **E**) and glutamine synthase (*red*; **A**, **D**) immunostaining in sections from the glaucomatous (**A–C**) and control (**D–F**) eye of a monkey with long-standing experimental glaucoma. (**C**, **F**) Merged images. *Blue*: DAPI-stained nuclei. C1q stained the processes of the Müller cells in the glaucomatous retina. ONL, outer nuclear layer; INL, inner nuclear layer.

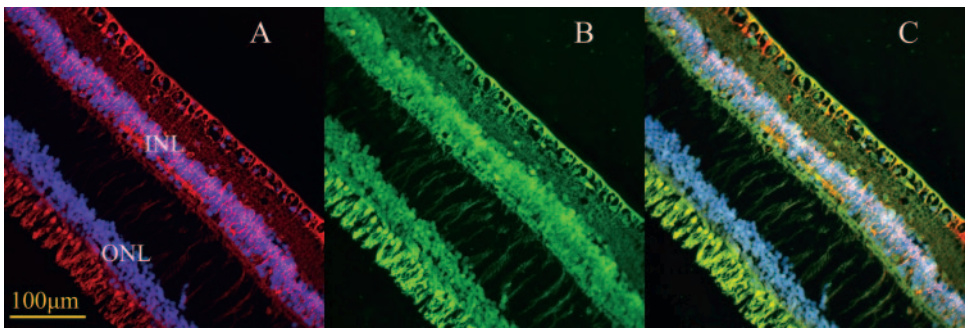

FIGURE 8. C1q (*green*; **B**) and glutamine synthase (*red*; **A**) immunostaining in sections from one of the glaucomatous eyes of a patient with long-standing glaucoma. (**C**) Merged image. *Blue*: DAPI-stained nuclei. C1q stained the processes of the Müller cells in the glaucomatous retina. ONL, outer nuclear layer; INL, inner nuclear layer.

It is interesting to note that the presence of increased neuronal C1q expression that occurs in Alzheimer's disease is also thought to be pathogenic in that neurodegenerative disease. It is tempting to speculate that glaucoma and Alzheimer's disease share another common mechanism that leads to neuronal death.

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