Current Eye Research, 32:917–922, 2007
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ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.1080/02713680701656343

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Complement Deposits on Ocular Tissues Adjacent to Sites of Inflammation

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ABSTRACT *Purpose*: The complement system plays important roles in a variety of chronic ocular diseases such as age-related macular degeneration. Here we examined the deposition of complement components in mouse eyes damaged by various mechanisms. *Methods*: Mouse eyes were damaged by light or by three models of inflammation, i.e., local transgenic expression of cytokines, interleukin-1 or -7, or by induction of experimental autoimmune uveitis. Eye tissues obtained from each model were immunostained with antibodies against complement components C1q, C3, and C4. *Results*: No complement deposition was seen in light damaged eyes, while in inflamed eyes we found complement deposition at sites of tissue damage and cellular infiltration. In addition to affected tissues, intense immunoreactivity against complement was unexpectedly observed in corneal tissues and lens capsule, despite lack of inflammation in these tissues. *Conclusion*: Our observations suggest that ocular tissues adjacent to inflammatory sites undergo changes that facilitate complement deposition.

KEYWORDS complement; cornea; experimental autoimmune uveitis; inflammation; lens capsule; light damage; transgenic mice

INTRODUCTION

It is becoming increasingly clear that immunologic mechanisms participate in a variety of ocular diseases many of which involve complement components.[1–5] Complement components contribute to pathogenic processes by damaging the tissue and being highly chemotactic and capable of facilitating neovascularization. Recently it was shown that complement components 3 and 5 (C3 and C5) are present in drusen in human eyes, suggesting that these inflammatory proteins may play a role in the development of age-related macular degeneration (AMD).[4] This notion has been in accord with the findings that polymorphisms in complement factor H increase the risk of developing AMD, while variants in factors B and C2 confer a significantly reduced risk of AMD.[6–9] C1q is upregulated in Mueller cells and the inner limiting membrane within the retina of animal models of glaucoma and in some eyes of patients with glaucoma.[5] The timing of this upregulation suggests that complement activity has a significant impact on the pathogenesis of glaucoma.[5] In a third major eye disease, diabetic retinopathy, extensive deposits of C3d and C5b-9 (membrane attack complex) were found in the choriocapillaris, under Bruch's membrane and in high concentration surrounding capillaries in ocular specimens with this disease.[10]

Received 13 July 2007
Accepted 29 August 2007

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This indicates that complement activation occurs to completion and may contribute to ocular tissue disease and visual impairment. The role played by complement in experimental autoimmune anterior uveitis (EAAU) was studied by comparing the course of intraocular inflammation in normal Lewis rats and rats that were complement depleted with cobra venom factor. The severity of the disease was reduced in complement-depleted rats, showing that complement, especially iC3b and its receptor (CR3), play a central role in the development of the disease.[3]

In our study, we examined different pathological conditions in mouse eyes to determine whether complement is involved in damage caused by different mechanisms. Experimental autoimmune uveitis (EAU), a model for human uveitis, is characterized by inflammatory changes mainly in the posterior segment, including accumulation of inflammatory cells in the vitreous, retina, and subretinal space. The retina can become detached and folded with loss of photoreceptors.[11] Eyes of interleukin (IL)-1 transgenic (Tg) mice, where IL-1 is expressed under control of the αA-crystallin promoter, include changes seen in all eye tissues and dense infiltrate of inflammatory cells.[12] IL-1 Tg animals could serve as models for rare diseases in humans in which inflammation develops due to excessive expression of cytokines, especially IL-1.[12] These conditions, named "auto-inflammatory diseases," respond to IL-1 inhibition.[13] IL-7 Tg mice expressing IL-7 under control of the αA-crystallin promoter develop cellular inflammatory infiltration and proteinaceous exudate in the anterior chamber and vitreous. Inflammatory cells can also be present in retina, vitreous, and choroid. Also characteristic of IL-7 Tg eyes are aggregates of lymphocytes in the limbus and the optic nerve pia and dura sheath.[12] Also included in our study was damage by exposure to light. This type of damage is of interest since a key feature of retinal dystrophies, and AMD is loss of visual cells by apoptosis, which can be studied through photoreceptor apoptosis induced by excessive exposure to light.[14]

When testing eyes damaged by the aforementioned mechanisms we found that no complement accumulation was seen in eyes damaged by light, while deposits of several complement components were found in tissues with severe inflammation. Unexpectedly, intense accumulation of complement was also seen in inflamed eyes on the lens capsule and corneal tissues, where no apparent damage was noted due to the inflammation.

MATERIALS AND METHODS

Models of Ocular Damage

Male or female mice between 6 and 12 weeks of age were used in the present study under protocols approved by the NEI Institutional Animal Care and User Committee. Eyes were damaged by the following procedures: (i) exposure of BALB/c mice to bright white light (5000 lux) for 25 hours, which causes death of retinal photoreceptors,[15] (ii) induction of EAU in B10.Br mice by immunization with 100 μg of whole interphotoreceptor retinoid binding protein (IRBP),[16] (iii) Tg expression under control of the αA-crystallin promoter of IL-1[17] or IL-7[12] in FVB/N mice. Healthy mice of the corresponding strains, age and sex matched, were used for controls. Eyes were collected immediately after light damage at 6 weeks of age from Tg mice or on day 21 post immunization from mice with EAU. The mice were euthanized with carbon dioxide and their eyes were placed in OCT and frozen for cryosectioning.

Immunochemistry and Imaging

Cryosections cut 8 μm thick were placed on Superfrost Plus (WWR, Batavia, IL) slides. Accumulation

FIGURE 1 **Complement deposition on ocular tissues of the posterior segment. Panels A, D, G, and J were stained with antibodies against complement components (C1q for panel G, or C3 for other panels). Panels B, E, H, and K were stained with antibodies against S-antigen (green) and anti-CD11b (red). Panels C, F, I, and L are negative controls, showing no staining with the IgG isotype control. Panels A, B, and C depict staining of a healthy FVB/N mouse eye that includes accumulation of C3 in the choroid (A) and trace staining for CD11b and for S-antigen (B). Please note that the photoreceptor layer is deleted in the normal FVB/N (rd) mouse used here as control for the Tg mouse eyes. Panels D, E, and F show the staining pattern in an eye with EAU that includes strong staining for C3 at the sites of inflammation, as well as Bruch's membrane (D). Staining for S-antigen is seen throughout the photoreceptor layer (partially covered by the DAPI stain), and strong staining for CD11b is seen on infiltrating inflammatory cells (E). Panels G, H, and I show the staining pattern of an IL-1 Tg eye that includes C1q deposited around photoreceptors, outer plexiform layer, and inflammatory sites (G). No S-antigen is detected, but intense staining for CD11b can be seen on cells in the vitreous as well as in the retina (H). Panels J, K, and L show the staining pattern in an IL-7 Tg eye that includes C3 accumulating at an area of the retina severely affected by the inflammatory process (J). No S-antigen is detected, whereas staining for CD11b can be seen in cells in the vitreous as well as in the retina (K). INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer; Ch, choroid; BM, Bruch's membrane.**

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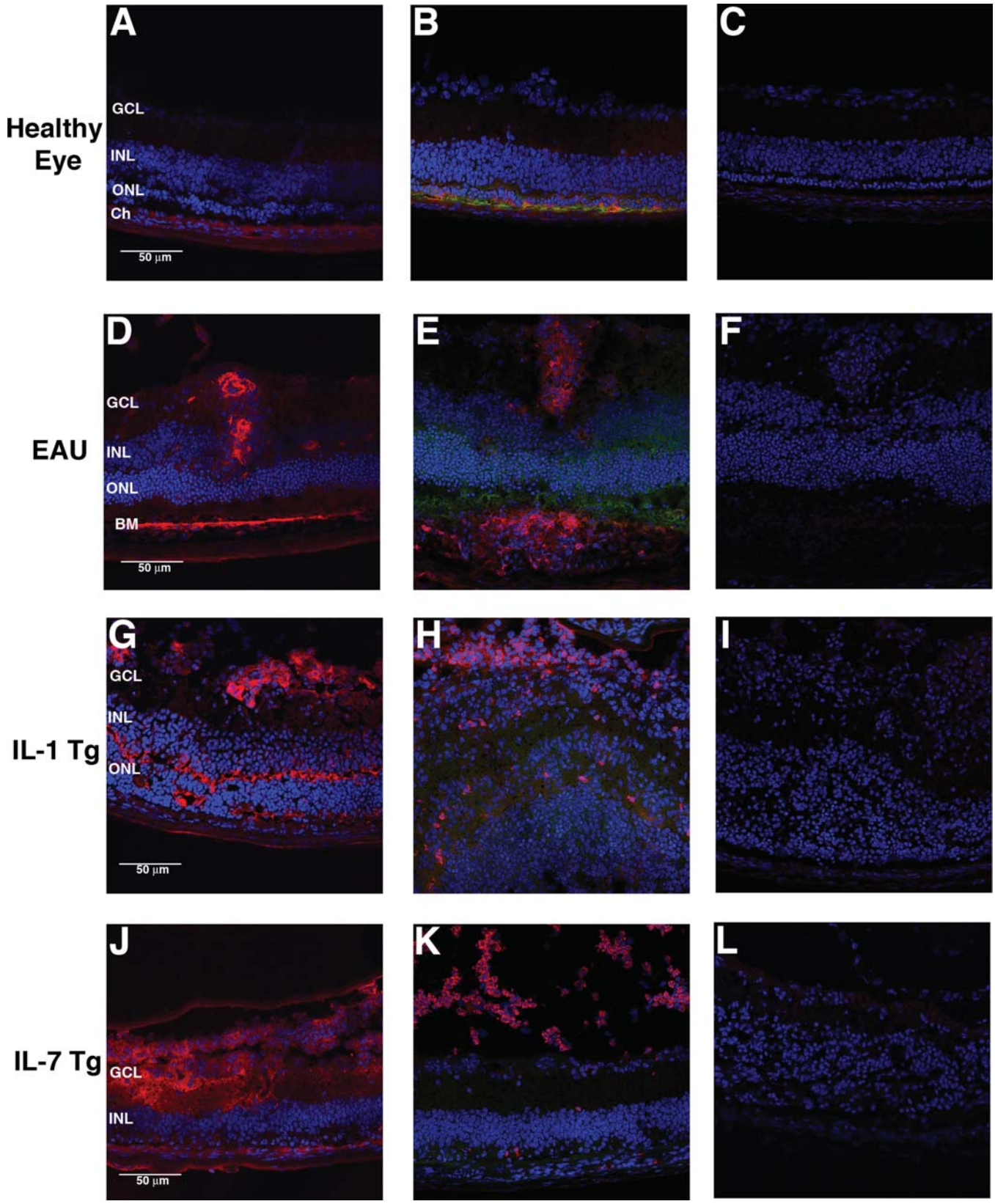

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of complement components was determined by immunofluorescence microscopy with monoclonal rat antibodies against mouse C1q, C3 (C3b/iC3b/C3c), C4 (Hycult Biotechnology, Netherlands). Rabbit anti-S-antigen[18] and rat anti-CD11b (Serotec, Raleigh, NC) antibodies were used for identifying the photoreceptor layer and monocytes/macrophages, respectively. Corresponding secondary antibodies used for detection included goat anti-rat IgG, labeled with Alexa Fluor© 568 and goat anti-rabbit IgG, labeled with Alexa Fluor© 488 (Invitrogen-Molecular Probes, Eugene, OR).

Slides with tissue sections were dried for 30 minutes at room temperature. The sections were then fixed with 4% paraformaldahyde for 10 minutes, followed by 3 washes with phosphate buffered saline (PBS). The sections were then incubated with 0.1 M glycine for 10 minutes followed by 3 washes with PBS. Slides were placed for 30 minutes in blocking solution consisting of 5% normal goat serum in ICC Buffer (0.5% BSA, 0.2% Tween 20, 0.05% Sodium Azide, in PBS, pH 7.3). Tissue sections were incubated with different primary antibodies diluted to 10 μg/ml in 2% normal goat serum for one hour and washed. The secondary antibody (6 μg/ml) and DAPI (1 mg/ml) were added to the tissue sections for 30 minutes, followed by washing with ICC buffer. Negative controls were incubated with rat IgG isotype controls (10 μg/ml), or without primary antibody during the primary antibody step. Specificity of the C3 antibody was confirmed by checking for immunoreactivity in C3–deficient tissues.

Slides were imaged on a Leica DMRXE SP2 Confocal Microscope (Leica, Bannockburn, IL).

RESULTS

The immunostaining patterns with antibodies against C1q and C3 were generally similar, whereas weaker staining was found with the antibody against C4. Therefore, the staining shown here were those with immunoreactivity to C1q or C3. All tested mouse eyes, including healthy eyes (Fig. 1A), had complement accumulation in the choroid, whereas only trace or no complement staining was seen in any of the other eye tissues of healthy mice (Figs. 1A, 2A, and 2D). On the other hand, complement components accumulated at the inflammatory foci in severely inflamed eyes, as detailed below.

Findings of interest include the following: no complement deposition was detected in retinas damaged by light (not shown). A unique observation in eyes with EAU was that complement accumulated in Bruch's membrane and the inflammatory sites (Fig. 1D). Eyes of IL-1 Tg mice showed the most intense inflammation. Complement deposition in these eyes was located in the outer plexiform layer as well as on infiltrating inflammatory cells (Fig. 1G). In eyes from IL-7 Tg mice, complement accumulation was evident in areas of damage and around infiltrating inflammatory cells (Fig. 1J).

In addition, we unexpectedly found complement deposition in the cornea and on the lens capsule of most inflamed eyes. The cornea and anterior segment in mice with EAU, shown in Figure 2B, did not display any signs of inflammation based on histological examination of the eyes. Eyes from IL-1 and IL-7 Tg mice may develop signs of inflammation and cellular infiltration in their corneas and anterior segments and these corneas were not shown here. Complement staining of the lens capsule is shown in Figure 2E. No inflammation or cellular infiltration was detected in the lens capsule of this IL-1 Tg mouse or of any mouse eye.

DISCUSSION

Similar patterns of deposition were seen in our study for C1q, C3, and C4 for each type of ocular damage, but the intensity of immunoreactivity against C4 was lower than the two other antibodies. Expression of complement by these ocular tissues themselves has yet to be determined and cannot be excluded as a possibility. The complement components accumulated around the inflammatory sites in the retina and vitreous (Figs. 1D, 1G, and 1J). This deposition of complement molecules was expected since the mechanisms that cause inflammation in these eyes also activate the complement cascade. The models of damage studied here are induced by different mechanisms, none of which is triggered directly by complement, but the complement components may enhance the damage initiated by the cytokines in the transgenic mice or the pathogenic process triggered by immunization of the mice in EAU. Eyes with EAU were used here on day 21 following immunization, i.e., a few days following the peak of the inflammatory process.[19] Eyes of Tg mice were examined at 6 weeks of age, when the inflammation is severe, but the ocular structures and various cell layers can be readily identified. In older Tg mice the inflammatory process may distort the ocular tissues and all layers.[12]

Unlike the anticipated deposition of complement on damaged tissues, it was unexpected to observe

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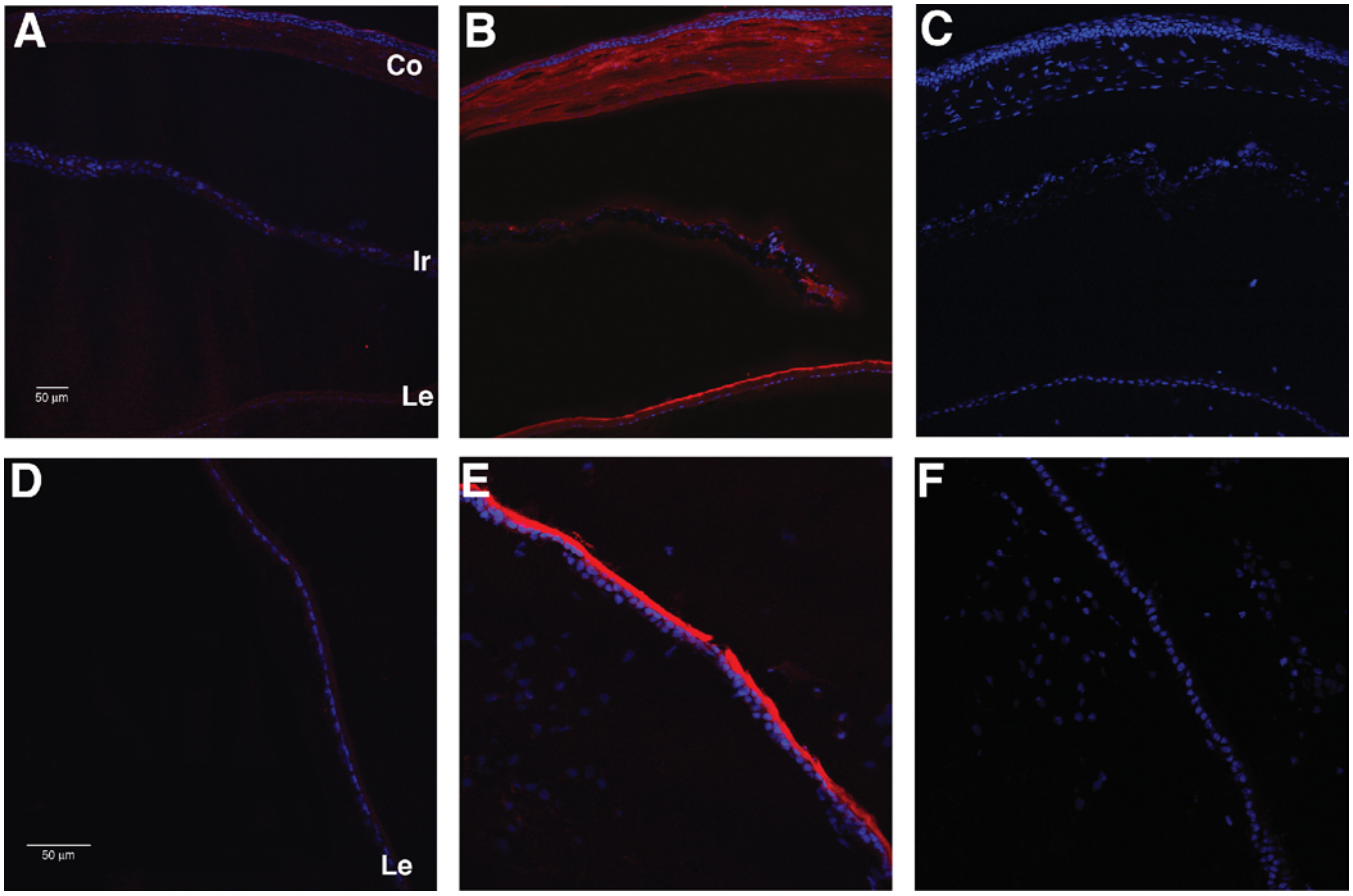

FIGURE 2 **Complement deposition on ocular tissues of the anterior segment. A, a section of the anterior segment of a healthy mouse eye showing essentially no accumulation of complement (C3) in the cornea (Co) or the lens capsule (Le). Iris is indicated by "Ir" and labeled structures correspond to same structures in Figs. 2B and 2C. B, a section of an eye with EAU, showing C3 accumulation in the uninflamed cornea and lens capsule. C, negative control of anterior segment of an eye with EAU, showing that the IgG isotype control does not interact with the tissue section. D, lens (Le) of a healthy mouse eye, showing no complement (C1q) deposition. E, lens of an IL-1 Tg mouse, showing intense C1q accumulation on the lens capsule. F, negative control of lens of an IL-1 Tg eye.**

complement localization in the cornea or lens capsule, where no inflammation developed (Figs. 2B and 2E). The mechanism responsible for the accumulation of complement in these apparently uninflamed tissues is not understood. We suggest that molecules released by inflammatory cells and inflamed tissues affect adjacent tissues not directly involved in the pathogenic process, rendering these structures susceptible to complement deposition.

Unlike eyes with inflammatory damage, no complement accumulation was found in eyes injured by intense light, despite the loss of photoreceptors in the eyes.[15] Lack of complement accumulation in these eyes could be attributed to the apoptotic mechanism that is involved in light damage, a mechanism that does not induce an inflammatory response.

In summary, complement accumulated in ocular tissues damaged by different inflammatory mechanisms. Interestingly, tissues not directly affected by inflammation, such as the cornea and lens capsule also displayed complement deposition, perhaps due to a change in the expression of complement receptors.

ACKNOWLEDGEMENTS

This research was funded by the Intramural Research Programs of the NEI, NIH. The authors would like to thank Dr. Robert N. Fariss for his continuous support and assistance with confocal microscopy and Mary Alice Crawford for her assistance with tissue preparation and sectioning.

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