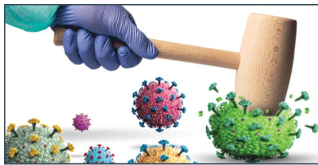

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Role of Complement and Complement Membrane Attack Complex in Laser-Induced Choroidal Neovascularization[1]

Puran S. Bora,[2] Jeong-Hyeon Sohn, Jose M. C. Cruz, Purushottam Jha, Hiroki Nishihori, Yali Wang, Sankaranarayanan Kaliappan, Henry J. Kaplan, and Nalini S. Bora

Choroidal neovascularization (CNV), or choroidal angiogenesis, is the hallmark of age-related macular degeneration and a leading cause of visual loss after age 55. The pathogenesis of new choroidal vessel formation is poorly understood. Although inflammation has been implicated in the development of CNV, the role of complement in CNV has not been explored experimentally. A reliable way to produce CNV in animals is to rupture Bruch's membrane with laser photocoagulation. A murine model of laser-induced CNV in C57BL/6 mice revealed the deposition of C3 and membrane attack complex (MAC) in the neovascular complex. CNV was inhibited by complement depletion using cobra venom factor and did not develop in $C3^{-/-}$ mice. Anti-murine C6 Abs in C57BL/6 mice inhibited MAC formation and also resulted in the inhibition of CNV. Vascular endothelial growth factor, TGF-β2, and β-fibroblast growth factor were elevated in C57BL/6 mice after laser-induced CNV; complement depletion resulted in a marked reduction in the level of these angiogenic factors. Thus, activation of complement, specifically the formation of MAC, is essential for the development of laser- induced choroidal angiogenesis in mice. It is possible that a similar mechanism may be involved in the pathophysiology of other angiogenesis essential diseases. *The Journal of Immunology,* **2005, 174: 491–497.**

Age-related macular degeneration (AMD)[3] is the leading cause of visual loss in individuals over age 55. Two major clinical phenotypes of AMD are recognized—a nonexudative (dry) type and an exudative (wet) type. Although choroidal neovascularization (CNV), which causes exudative type AMD, occurs in a minority of patients with AMD, ~10% of AMD cases are of the exudative type. CNV is responsible for the sudden and disabling loss of central vision (1–5).

CNV is a complex biological process and the pathogenesis of new choroidal vessel formation is not completely understood (6–11). Several factors, such as inflammation (3, 12–14), ischemia (13), and local production of angiogenic factors (15, 16), are thought to be important in the pathogenesis of CNV. Recent studies have identified the macrophage as an important component of laser-induced CNV response (17–19).

Although inflammation has been implicated in the development of CNV, the role of complement has not been explored. The complement system is a major component of innate immunity and plays a central role in the host defense against infection (20–22). Membrane attack complex (MAC), the final product of the activated complement cascade has been reported to release growth factors such as β-fibroblast growth factor (β-FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor from various nucleated cells (23–26). These reports suggest that MAC-mediated release of growth factors from nucleated cells may be a pathogenic mechanism in angiogenesis. A reliable way to produce CNV in animals is to rupture Bruch's membrane with laser photocoagulation (27, 28). In the present study, we used the mouse model of laser-induced CNV to investigate the role of the complement system, and particularly MAC formation in the choroidal angiogenesis.

Materials and Methods

Animals

Male C57BL/6 mice (6–8 wk old), C3-deficient mice ($C3^{-/-}$), and their wild-type control ($(129 \times C57BL/6)F_1$) were purchased from The Jackson Laboratory. This study was approved by the Institutional Animal Care and Use Committee of University of Louisville.

Induction of CNV in mice

Animals were divided into four groups. CNV was induced by laser photocoagulation in C57BL/6 mouse (group 1; $n = 10$; complement sufficient) with the krypton red laser (50-μm spot size; 0.05-s duration; 250 mW) as previously described by us (29, 30). Three laser spots were placed in each eye close to the optic nerve. In group 2, C57BL/6 mice ($n = 10$) were treated i.p. with 4 U of cobra venom factor (CVF; Quidel) 2 days before laser photocoagulation and every day after laser treatment. We refer to these animals as "complement depleted" throughout this paper. Group 3 had $C3^{-/-}$ mice ($n = 10$), and group 4 consisted of 10 wild-type control ($(129 \times C57BL/6)F_1$) for the C3-deficient mice. Laser photocoagulation in all four groups was performed as described above. These experiments were repeated five times.

Measurement of CNV and CNV lesions

Seven days after laser treatment, all animals were perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran (FITC-dextran; average molecular mass, 2×10^6; Sigma-Aldrich) and sacrificed. The eyes were harvested and fixed in 10% phosphate-buffered formalin, and retinal pigment epithelium (RPE)-choroid-scleral flat mounts were prepared as previously described (29, 30). RPE-choroid-scleral flat mounts were stained for elastin using a mAb specific for elastin (1.0 mg/ml; 1/200 dilution; Sigma-Aldrich) followed by a Cy3-labeled secondary Ab (1.0 mg/ml; 1/200 dilution; Sigma-Aldrich). The incidence and the size of CNV

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[3] Abbreviations used in this paper: AMD, age-related macular degeneration; CNV, choroidal neovascularization; MAC, membrane attack complex; β-FGF, β-fibroblast growth factor; VEGF, vascular endothelial growth factor; CVF, cobra venom factor; RPE, retinal pigment epithelium; F, forward; R, reverse; CH_{50}, total complement hemolytic activity.

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was determined by confocal microscopy. The size of the CNV complex was graded by morphometric analysis of the images (MetaMorph Image Analysis software package; Universal Imaging) obtained from confocal microscopy (29, 30).

Abs and treatment

Polyclonal Ab to mouse C6 (purified rabbit IgG) was obtained from Cell Sciences. C57BL/6 mice were divided into two groups. Animals in group 1 (n = 13) received 40 μg of anti-murine C6 i.p. daily for a total of 8 days. Control animals (group 2; n = 11) received a similar treatment with purified polyclonal normal rabbit IgG (BD Pharmingen). On day 8, animals in both groups were treated with laser to induce CNV as described above. The animals were sacrificed on day 7 post-laser treatment; measurement of CNV and CNV lesions was also performed as described above. Animals (n = 3 each group) were sacrificed at 72 h post-laser treatment, and flat mounts prepared from both Ab-treated and control animals were stained for MAC as described below. Both anti-C6-treated and control (normal rabbit IgG-injected) mice were bled on day 8 of Ab treatment for determination of serum complement hemolytic activity. Hemolytic assay was performed using EZ Complement CH_{50} Test (Diamedix) according to the manufacturer's instruction with some modifications. Briefly, 150 μl of Ab-sensitized sheep erythrocytes was incubated with rat serum sequentially diluted to give a total volume of 200 μl at 37°C for 60 min. Serum obtained from normal C57BL/6 mice was used to determine the 100% value for complement-dependent serum hemolytic activity.

Immunohistochemical studies

Flat mounts were stained for C3 and MAC. A polyclonal Ab (raised in rabbit; 1.0 mg/ml) reactive with rat/mouse C9 was used to stain for mouse MAC, and the IgG fraction of goat anti-mouse C3 (6.0 mg/ml; ICN) was used for C3 staining. These Abs were used at 1/200 dilution. Cy3-conjugated anti-rabbit IgG and anti-goat IgG obtained from Sigma-Aldrich were used as the secondary Abs for MAC and C3 staining, respectively. Control stains were performed with normal rabbit or goat serum at concentrations similar to those of the primary Abs. Additional controls consisted of staining by omission of the primary or secondary Ab (31). The flat mounts were examined with a confocal microscope (LSM510; Zeiss).

RT-PCR analysis

Twenty laser spots were placed in each eye of complement-sufficient and complement-depleted (CVF-treated) C57BL/6 mice as described above. Animals from each group (n = 10/each time point) were sacrificed at days 1, 3, 5, and 7 post-laser treatment; RPE-choroid-scleral tissues harvested from the enucleated eyes were pooled separately for each time point, and total RNA was prepared using SV Total RNA Isolation kit (Promega). Equal amounts of the total RNA (0.2 μg) were used to detect the mRNA levels of GAPDH, VEGF, TGF-β2, and β-FGF by RT-PCR using the reagents purchased from Applied Biosystems. The sense and antisense oligonucleotide primers were synthesized at Integrated DNA Technologies, and PCR used 30 cycles. The negative controls consisted of omission of RNA or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 2% agarose gel and were examined by using the Molecular Analyst/PC program (Bio-Rad). These experiments were repeated three times with similar results. RT-PCR was conducted using the following primers: GAPDH (forward (F), 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; reverse (R), 5′-CATGTAG GCCATGAGGTCCACCAC-3′); VEGF (F, 5′- GCGGGCTGCCTCGCA GTC-3′; R, 5′-TCACCGCCTTGGCTTGTCAC- 3′); β-FGF (F, 5′-AGCGGCTCTACTGCAAGAAC-3′; R, 5′-TCGTTTCAGTGCCACAT ACC-3′); TGF-β2 (F, 5′-CCAAAGACTTAACATCTCCCACC-3′; R, 5′-GTTCGATCTTGGGCGTATTTC-3′).

ELISA

Twenty laser spots were placed in each eye of complement-sufficient and complement-depleted C57BL/6 mice as described above. Animals from each group (n = 10/each time point) were sacrificed at days 1, 3, 5, and 7 post-laser treatment, and RPE-choroid-scleral tissues were harvested from the enucleated eyes. RPE-choroid-scleral tissues for different time points were pooled separately. Pooled tissue was homogenized and solubilized in ice-cold PBS containing protease inhibitors (32).

The samples were assayed (in triplicate) for β-FGF and VEGF proteins using human β-FGF and mouse VEGF ELISA kits (R&D Systems). These experiments were repeated three times with similar results, and the data are represented as mean ± SD.

Statistics

Differences between groups were evaluated by Student's t test.

Results

Role of complement in the development of laser-induced CNV

The role of complement in the development of CNV was investigated by using C57BL/6 mice depleted of systemic complement by

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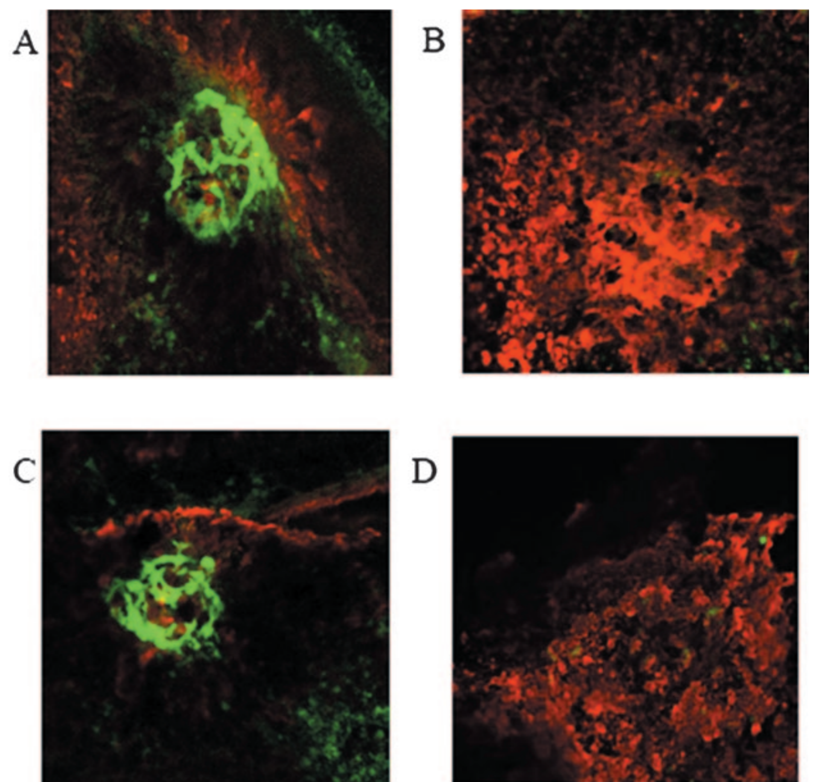

FIGURE 1. Laser-induced subretinal neovascular complex in C57BL/6 and $C3^{-/-}$ mice. Mice were anesthetized and perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran. The flat mounts were stained for elastin by using elastin Ab and Cy3-conjugated secondary Ab, and were examined by confocal microscope. *A*, Confocal micrograph of the neovascular complex in C57BL/6 mice showing the new vessels (green). Exposed Bruch's membrane with elastin stained red; otherwise, intact RPE obscures Bruch's membrane. *B*, Neovascular complex did not develop in the laser spots in C57BL/6 mice treated with CVF. *C* and *D*, Angiogenesis developed in wild-type controls ((129 × C57BL/6)F_1) for $C3^{-/-}$ mice (*C*), whereas no CNV was observed in $C3^{-/-}$ mice (*D*) (×2500).

Table I. *Effect of complement on laser-induced CNV in mice*[a]

Mice	No. of Mice	Treatment	Laser Spots/Eye	Total Spots	CNV-Positive Spots (No. (%))	CNV-Negative Spots (No. (%))
C57BL/6	50	None	3	300	289 (96)	11 (4)
C57BL/6	50	CVF	3	300	10 (3)	290 (97)
C57BL/6	8	Anti-C6	3	48	13 (27)	35 (73)
C57BL/6	8	NR-IgG	3	48	45 (94)	3 (6)
$C3^{-/-}$	50	None	3	300	16 (5)	284 (95)
WT control	50	None	3	300	292 (97)	8 (3)

[a] Animals were sacrificed on day 7 post-laser treatment. NR-IgG, Normal rabbit IgG; $C3^{-/-}$, C3-deficient mice; WT, wild type. Student's *t* test of the data showed $p < 0.001$.

CVF. Our results are summarized in Fig. 1, *A* and *B*, and Table I. Flat-mount analysis revealed that the incidence of CNV in complement-sufficient C57BL/6 mice (Fig. 1*A*) was 98%. However, the in vivo depletion of systemic complement with CVF markedly reduced ($p < 0.001$) the development of CNV (Fig. 1*B*) to 3%. A total complement hemolytic activity (CH_{50}) assay (33) confirmed the absence of functionally active complement in CVF-injected mice. On days 1, 3, 5, and 7, CH_{50} levels in CVF-treated mice were 2, 3, 3, and 2%, respectively, compared with control (non-CVF-treated) mice. CH_{50} levels in control animals were 100% at these time points. No changes were observed on light-microscopic examination of the heart, kidney, and liver of these animals (data not shown). Each experiment was repeated five times with similar results.

Role of C3 in the development of laser-induced CNV

Using $C3^{-/-}$ mice, we next explored whether the development of CNV required C3. Laser photocoagulation induced CNV in the wild-type ((129 × C57BL/6)F_1) control (incidence, 98%; Table I; Fig. 1*C*), but not in the $C3^{-/-}$ mouse (incidence, 5%; *D*). No changes were again observed on histologic examination of the heart, kidney, and liver of these animals (data not shown). Each experiment was repeated five times with similar results.

Deposition of C3 in CNV complex

Flat mounts of the CNV complex were stained for C3. The neovascular complex stained for C3 in C57BL/6 (Fig. 2*A*) and (129 × C57BL/6)F_1 (*C*) mice on day 1 post-laser treatment. Similar results were observed on days 3 and 5 postlaser with weak staining on day 7 (data not shown). In contrast, no C3 staining was observed in the laser spots of CVF-treated mice (Fig. 2*B*), as well as $C3^{-/-}$ mice on day 1 (*D*) through day 7 (data not shown). No staining was observed in the control sections stained without the primary Ab (data not shown).

Deposition of MAC in CNV complex

Flat mounts of the CNV complex were also stained for MAC. A pattern similar to C3 deposition was observed in laser spots stained for MAC in C57BL/6 (Fig. 3, *A*, *C*, and *E*) and (129 × C57BL/6)F_1 (data not shown) mice on days 1, 3, and 5 post-laser treatment. Very weak staining for MAC was noted on day 7 (Fig. 3*E*). In contrast, no MAC staining was observed in the laser spots of complement-depleted mice (CVF-treated; Fig. 3, *B*, *D*, and *F*) and $C3^{-/-}$ (data not shown) mice from day 1 through day 7. No staining was observed in the control sections stained without the primary Ab (data not shown).

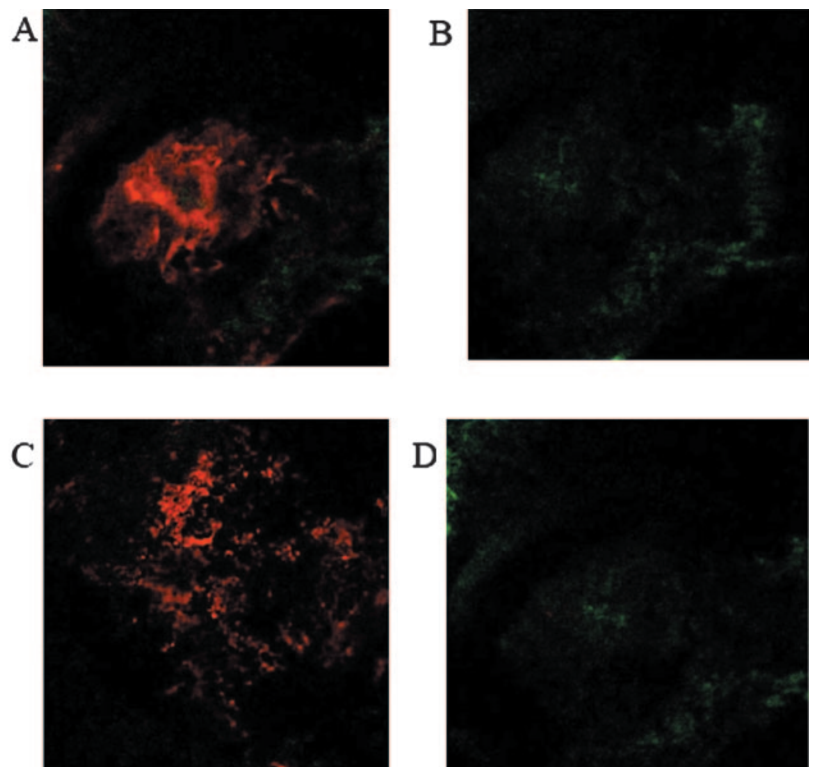

FIGURE 2. Staining for C3 in laser-induced neovascular complex in C57BL/6 and $C3^{-/-}$ mice, 24 h after laser treatment. Purified IgG fraction of goat antiserum to mouse C3 was used as the primary Ab, and Cy3-conjugated anti-goat IgG served as the secondary Ab. *A* and *C*, CNV lesions stained red for C3 in C57BL/6 (*A*) and $C3^{-/-}$ wild-type control mice (*C*). *B* and *D*, No staining for C3 was observed in C57BL/6 mice treated with CVF (*B*) and $C3^{-/-}$ mice (*D*) (×2500).

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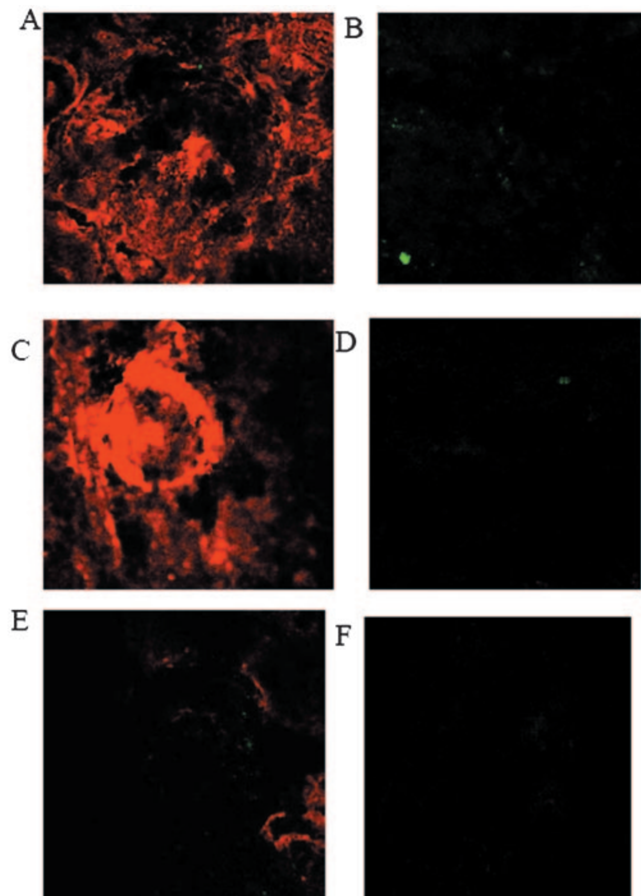

FIGURE 3. Staining for MAC in laser-induced neovascular complex in C57BL/6 mice on day 1 (*A* and *B*), day 3 (*C* and *D*), and day 7 (*E* and *F*) after laser treatment. Purified IgG fraction of rabbit antiserum to mouse C9 was used as the primary Ab, and Cy3-conjugated anti-rabbit IgG served as the secondary Ab. Laser spots (CNV lesions) stained red for MAC in C57BL/6 at days 1 and 3 postlaser (*A* and *C*) with very little staining at day 7 (*E*). No staining for MAC was observed in CVF-treated C57BL/6 mice on day 1 (*B*) day 3 (*D*), and day 7 (*F*). *A*, *B*, and *D–F*, ×2500; *C*, ×2000.

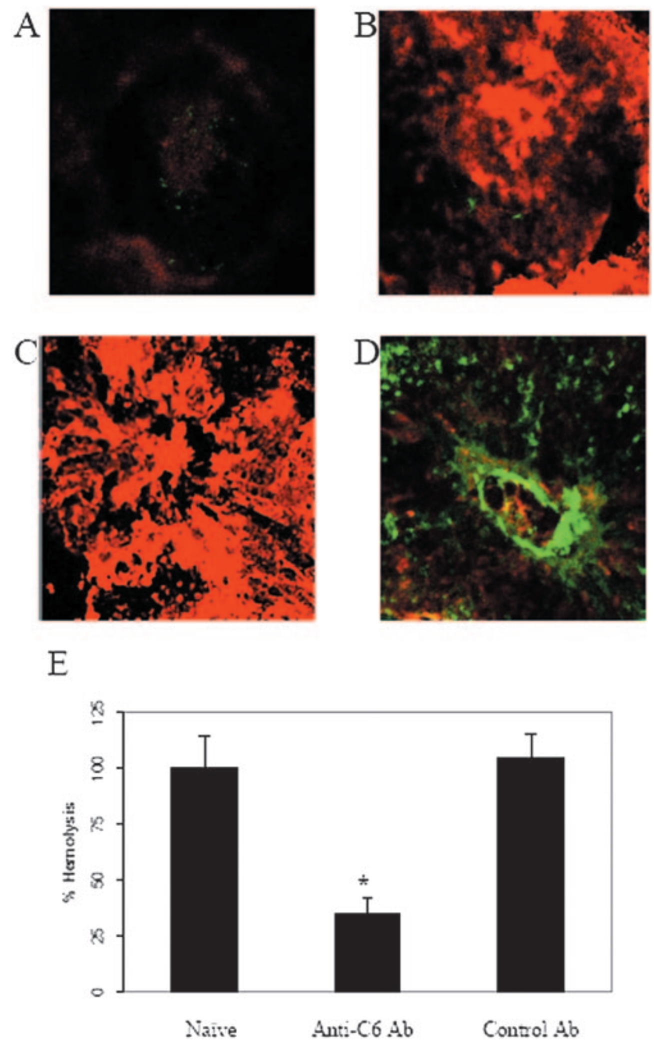

FIGURE 4. Effect of anti-murine C6 on MAC deposition (*A* and *B*), formation of CNV complex (*C* and *D*), and serum complement hemolytic activity (*E*). MAC staining was done on day 3 postlaser. *A* and *B*, Very weak MAC deposition was observed in C57BL/6 mice treated with anti-C6 (*A*), in contrast to the intense red staining for MAC in control animals (*B*). *C* and *D*, On day 7, the CNV complex (green stain) did not develop in anti-C6-treated mice (*C*), in contrast to control mice (*D*). Exposed Bruch's membrane stained red for elastin (×2500). *E*, Anti-C6-treated animals had significantly (*, $p < 0.0001$) reduced (65–70%) complement hemolytic activity compared with naive animals and IgG-injected controls.

Effect of anti-C6 on MAC formation and the induction of laser-induced CNV

To further examine the role of MAC (C5b-9), we used polyclonal Abs that inhibit the complement cascade at C6, thus blocking the generation of C5b-9. Our results showed that systemic administration of the anti-C6 polyclonal Abs dramatically reduced the deposition of MAC in the laser spots (Fig. 4*A*) on day 3 post-laser treatment, whereas the laser spots stained very strongly for MAC in the control (normal rabbit IgG-treated) mice (*B*). Our results also showed that C6 inhibition had a dramatic effect on the induction and development of laser-induced CNV (Table I). CNV was significantly ($p < 0.001$) reduced in anti-C6-treated mice (Fig. 4*C*; Table I) compared with control (rabbit IgG-treated) animals (*D*). Inhibition of complement by anti-C6 polyclonal Abs was confirmed by measurement of complement-dependent serum hemolytic activity. At the time of induction of CNV (i.e., time of laser treatment), C6 Ab-treated animals had significantly ($p < 0.0001$) reduced (65–70%) serum hemolytic activity relative to naive and control rabbit IgG-injected mice (Fig. 4*E*). These results established a central role for complement activation and MAC formation in the induction and development of laser-induced CNV in mice.

Effect of complement on the ocular production of VEGF, TGF-β2, and β-FGF

Laser-induced CNV in the mouse is associated with the increased production of several angiogenic factors within the retina and RPE. Thus, we studied the relationship between activation of the complement cascade and the intraocular expression of VEGF, TGF-β2, and β-FGF in this model. Using RT-PCR, we detected low levels of VEGF (716, 644, and 512 bp), TGF-β2 (684 bp), and β-FGF (298 bp) mRNA at 24 h in laser-treated C57BL/6 mice (Fig. 5*A*). We previously noted that complement activation occurred within 24 h of laser treatment with the marked deposition of MAC at the site of laser injury at 24 h (Fig. 3*A*). VEGF, TGF-β2, and β-FGF transcripts increased on days 3 and 5, and returned to basal levels on day 7 (Fig. 5*A*). This was similar to the pattern we observed for MAC deposition. In contrast, the mRNA of these growth factors did not change and remained at low basal levels through day 7 in CVF-treated mice (Fig. 5*A*). A strong band at 983 bp for GAPDH

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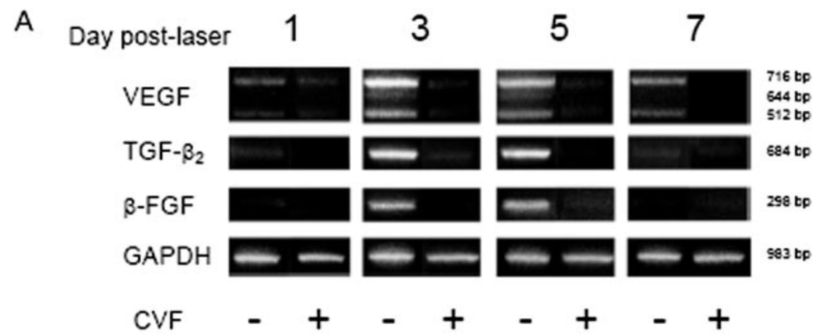

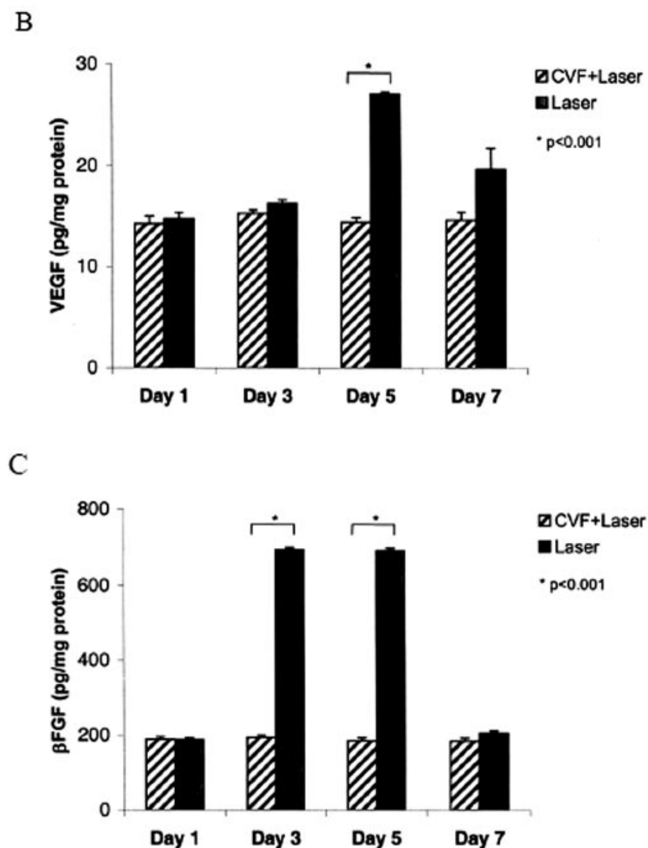

FIGURE 5. Effect of complement on intraocular VEGF, TGF-β2, and β-FGF mRNA expression (*A*) and protein (*B* and *C*) during laser-induced CNV in C57BL/6 mice. *A*, The figure shows ethidium bromide-stained bands for PCR product after UV exposure. In the presence of complement, VEGF, TGF-β2, and β-FGF transcripts increased at day 3, remained at that level on day 5, and returned to basal levels on day 7. However, in the absence of complement (CVF-treated mice), the levels of these angiogenic factors remained at basal constitutive levels. *B* and *C*, Effect of complement on VEGF and β-FGF protein production during laser-induced CNV in C57BL/6 mice. On day 5, the levels of VEGF were significantly increased ($p < 0.001$) in complement-sufficient mice compared with CVF-treated animals (*B*). A marked increase ($p < 0.001$) in β-FGF protein levels was observed on days 3 and 5 in complement-sufficient mice compared with CVF-treated animals (*C*).

indicated equal amounts of RNA in each lane (Fig. 5*A*). No band was seen in the controls without RNA or reverse transcriptase (data not shown).

Using ELISA, low levels of VEGF protein were observed on days 1 and 3 postlaser with a significant increase on day 5 ($p < 0.001$) in C57BL/6 mice (Fig. 5*B*). β-FGF protein remained at constitutive levels on day 1 but increased significantly ($p < 0.001$) on days 3 and 5 (Fig. 5*C*). In contrast, the levels of both proteins remained at basal levels throughout day 7 in CVF-treated (complement-deficient) C57BL/6 mice (Fig. 5, *B* and *C*). These findings suggest that complement activation and MAC (C5b-9) deposition are important for the production and release of angiogenic growth factors in laser-induced CNV. Our results of RT-PCR and ELISA demonstrated that the production of angiogenic factors—VEGF, TGF-β2, and β-FGF—is temporarily regulated and dependent on the presence and activation of the complement system.

Discussion

Angiogenesis is a fundamental process occurring during embryonic development but is a characteristic of various pathological conditions in the adult (34, 35). Choroidal angiogenesis (i.e., CNV) associated with AMD is the leading cause of visual loss in individuals over age 55 (1). In the present study, we have used a murine model of laser-induced CNV (3, 27–30) to understand the role of complement in choroidal angiogenesis.

First, we demonstrated that complement was essential for the development of CNV in C57BL/6 mice after laser photocoagulation. C57BL/6 mice depleted of complement with CVF did not develop CNV. Because C3 is a key component of complement, and has been demonstrated to play an important role in various immune responses (20–22, 31), we investigated whether it was required for the development of CNV. We report here for the first time that $C3^{-/-}$ mice were unable to develop CNV after laser photocoagulation.

We then focused our attention on the formation and deposition of the C5b-9 MAC in CNV lesions. MAC has previously been reported to be spontaneously and continuously deposited on self-tissue in small amounts under normal conditions and in larger quantities under various pathological conditions (32, 36–38). Using the mouse model of laser-induced CNV, we observed that the neovascular complex stained very strongly for C3 and MAC in complement-sufficient C57BL/6 mice. In contrast, laser spots in $C3^{-/-}$ mice, as well as

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complement-depleted (CVF-treated) C57BL/6 mice, did not stain for C3 or MAC. These results showed a correlation between the presence of complement, MAC deposition, and choroidal new vessel formation following laser photocoagulation.

The importance of MAC in the development of CNV was demonstrated by the in vivo inhibition of complement C6 with anti-murine C6 polyclonal Abs. The systemic administration of anti-C6 Abs inhibited the in vivo formation of MAC and markedly reduced both the incidence and development of CNV in C57BL/6 mice after laser treatment. Although anti-C6 Ab inhibits the sequential assembly of the C5b-9 (MAC) complex, the generation of C3a and C5a is not affected (20–22). Thus, our results suggest that, without the formation of MAC at the site of injury, laser-induced choroidal angiogenesis will not occur. Using anti-C6 or anti-C5 Abs, similar observations were made in experimental autoimmune myasthenia gravis (39), collagen-induced arthritis (40), and lupus-like autoimmune disease in NZB/WF1 mice (41).

Because we established a critical role for MAC deposition in the development of laser-induced CNV, we asked whether the generation of the growth factors observed to be important in the development of CNV (42–47) was affected by MAC. We confirmed that within 3 days of laser photocoagulation, there was an increased production of VEGF, TGF-β2, and β-FGF in CNV lesions. However, when the animals were complement depleted with CVF, so that no MAC deposition occurred, the levels of these growth factors remained low. Interestingly, in complement-sufficient animals, the levels of these growth factors returned to baseline level at day 7 postlaser, because at this time point, very little MAC deposition was observed in the CNV complex of these animals. Thus, the development of choroidal angiogenesis following laser photocoagulation is dependent on the deposition of MAC and the subsequent generation and secretion of angiogenic factors. The release of several growth factors, such as β-FGF and platelet-derived growth factor, from epithelial and endothelial cells has been observed following the deposition of MAC (23–26).

Only recently has it become apparent that complement is important in AMD (48–51). Using the in vitro techniques, some investigators have suggested that impaired macrophage mobilization and recruitment allow the accumulation of C5a (and IgG), which induced VEGF production (19). Our results reported here clearly demonstrate that MAC formation and deposition are critical for the increased production of growth factors—VEGF, β-FGF, and TGF-β2—which eventually leads to the development of laser-induced CNV.

In conclusion, our studies describe the first direct role of complement activation and MAC formation in the laser-induced choroidal angiogenesis. On the basis of the results presented here, we propose the following potential mechanism defining the role of complement in CNV: Complement activation (via the classical or alternative pathway) in the posterior segment of the eye leads to increased formation and deposition of MAC on RPE and/or choroid. This results in transient changes in the membrane permeability followed by induction and release of growth factors. Released growth factors cause abnormal proliferation of choroidal endothelial cells leading to the development of CNV. Thus, in the absence of complement, CNV will not occur and complement inhibition may be used as a suitable therapeutic tool in the treatment of CNV. It is possible that a similar mechanism may be involved in the pathogenesis of other neovascular diseases such as diabetic retinopathy, arteriosclerosis, arthritis, and cancer.

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References

1. Ferris, F. L., S. L. Fine, and L. Hyman. 1984. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch. Ophthalmol. 102:1640.*
2. Fine, S. L., J. W. Berger, M. G. Maguire, and A. C. Ho. 2000. Age-related macular degeneration. *N. Engl. J. Med. 342:483.*
3. Campochiaro, P. A. 2000. Retinal and choroidal neovascularization. *J. Cell. Physiol. 184:301.*
4. Chang, B., L. A. Yannuzzi, I. D. Ladas, D. R. Guyer, J. S. Slakter, and J. A. Sorenson. 1995. Choroidal neovascularization in second eyes of patients with unilateral exudative age-related macular degeneration. *Ophthalmology 102:1380.*
5. Smith, W., J. Assink, R. Klein, P. Mitchell, C. C. Klaver, B. E. Klein, A. Hofman, S. Jensen, J. J. Wang, and P. T. de Jong. 2001. Risk factors for age-related macular degeneration: pooled findings from three continents. *Ophthalmology 108:697.*
6. Macular Photocoagulation Study Group. 1991. Argon laser photocoagulation for neovascular maculopathy: five-year results from randomized clinical trials. *Arch. Ophthalmol. 9:1109.*
7. Evans, J. R. 2001. Risk factors for age-related macular degeneration. *Prog. Retinal Eye Res. 20:227.*
8. Hawkins, B. S., A. Bird, R. Klein, and S. K. West. 1999. Epidemiology of age-related macular degeneration. *Mol. Vis. 5:26.*
9. La Cour, M., J. F. Kilgaard, and M. H. Nissen. 2002. Age-related macular degeneration: epidemiology and optimal treatment. *Drugs Aging 19:101.*
10. Husain, D., B. Ambati, A. P. Adamis, and J. W. Miller. 2002. Mechanisms of age-related macular degeneration. *Ophthalmol. Clin. North Am. 15:87.*
11. Yates, J. R., and A. T. Moore. 2000. Genetic susceptibility to age-related macular degeneration. *J. Med. Genet. 37:83.*
12. Jackson, J. R., M. P. Seed, C. H. Kircher, D. A. Willoughby, and J. D. Winkler. 1997. The codependence of angiogenesis and chronic inflammation. *FASEB J. 11:457.*
13. D'Amore, P. A. 1994. Mechanisms of retinal and choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci. 35:3874.*
14. Penfold, P. L., M. C. Madigan, M. C. Gillies, and J. M. Provis. 2001. Immunological and aetiological aspects of macular degeneration. *Prog. Retinal Eye Res. 20:385.*
15. Yi, X., N. Ogata, M. Komada, C. Yamamoto, K. Takahashi, K. Omori, and M. Uyama. 1997. Vascular endothelial growth factor expression in choroidal neovascularization in rats. *Graefes Arch. Clin. Exp. Ophthalmol. 235:313.*
16. Pierce, E. A., R. L. Avery, E. D. Foley, L. P. Aiello, and L. E. Smith. 1995. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc. Natl. Acad. Sci. USA 92:905.*
17. Sakurai, E., A. Anand, B. A. Ambati, N. V. Rooijen, and J. Ambati. 2003. Macrophage depletion inhibits experimental choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci. 44:3578.*
18. Espinosa-Heidmann, D. G., I. J. Suner, E. P. Hernandez, D. Monroy, K. G. Csaky, and S. W. Cousins. 2003. Macrophage depletion diminishes lesion size and severity in experimental choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci. 4:3586.*
19. Ambati, J., A. Anand, S. Fernandez, E. Sakurai, B. C. Lynn, W. A. Kuziel, B. J. Rollins, and B. K. Ambati. 2003. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat. Med. 9:1390.*
20. Atkinson, J. P., and M. M. Frank. 1980. Complement. In *Clinical Immunology*. C. W. Parker, ed. Saunders, Philadelphia, PA, p. 219.
21. Frank, M. M., and L. F. Fries. 1991. The role of complement in inflammation and phagocytosis. *Immunol. Today 12:322.*
22. Thomlinson, S. 1993. Complement defense mechanisms. *Curr. Opin. Immunol. 5:83.*
23. Morgan, B. P. 1992. Effects of the membrane attack complex of complement of nucleated cells. *Curr. Top. Microbiol. Immunol. 178:115.*
24. Halperin, J. A., A. Taratuska, and A. Nicholson-Weller. 1993. Terminal complement complex C5b-9 stimulates mitogenesis in 3T3 cells. *J. Clin. Invest. 91:1974.*
25. Benzaquen, L. B., A. Nicholson-Weller, and J. A. Halperin. 1994. Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. *J. Exp. Med. 179:985.*
26. Cybulsky, A. V., T. Takano, J. Papillon, and A. J. McTavish. 1999. Complement C5b-9 induces receptor tyrosine kinase transactivation in glomerular epithelial cells. *Am. J. Pathol. 155:1701.*
27. Miller, H., B. Miller, T. Ishibashi, and S. J. Ryan. 1990. Pathogenesis of laser induced choroidal subretinal neovascularization. *Invest. Ophthalmol. Vis. Sci. 31:899.*
28. Kimura, H., T. Sakamoto, D. R. Hinton, C. Spee, Y. Ogura, and S. J. Ryan. 1995. A new model of subretinal neovascularization in the rabbit. *Invest. Ophthalmol. Vis. Sci. 36:2110.*
29. Kaplan, H. J., M. A. Leibole, T. H. Tezel, and T. A. Ferguson. 1999. Fas-ligand (CD95 ligand) controls angiogenesis beneath retina. *Nat. Med. 5:292.*
30. Bora, P. S., Z. Hu, T. H. Tezel, J. H. Sohn, S. G. Kang, J. M. Cruz, N. S. Bora, A. Garen, and H. J. Kaplan. 2003. Immunotherapy for choroidal neovascularization in a laser-induced mouse model simulating exudative (wet) macular degeneration. *Proc. Natl. Acad. Sci. USA 100:2679.*
31. Sohn, J.-H., P. S. Bora, and N. S. Bora. 2003. Tolerance is dependent on complement C3 fragment iC3b binding to antigen-presenting cells. *Nat. Med. 9:206.*

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32. Sohn, J.-H., H. J. Kaplan, H.-J. Suk, P. S. Bora, and N. S. Bora. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. *Invest. Ophthalmol. Vis. Sci. 41:3492.*
33. Kabat, E. A., and M. M. Mayer. 1961. Complement and complement fixations. In *Experimental Immunochemistry.* A. Kabat and M. Mayer, eds. Thomas, Springfield, IL, p. 133.
34. Liekens, S., E. De Clercq, and J. Neyts. 2001. Angiogenesis: regulators and clinical applications. *Biochem. Pharm. 61:253.*
35. Hyder, S. M., and G. M. Stancel. 1999. Regulation of angiogenic growth factors in the female reproductive tract by estrogens and progestins. *Mol. Endocrinol. 13:806.*
36. Koffler, D., G. Biesecker, B. Noble, G. A. Andres, and A. Martinez-Hernandez. 1983. Localization of the membrane attack complex (MAC) in experimental immune complex (MAC) in experimental immune complex glomerulonephritis. *J. Exp. Med. 157:1885.*
37. Acosta, J., J. Hettinga, R. Fluckiger, N. Krumrei, A. Goldfine, L. Angarita, and J. Halperin. 2000. Molecular basis for a link between complement and the vascular complications of diabetes. *Proc. Natl. Acad. Sci. USA 17:5450.*
38. Gerl, V. B., J. Bohl, S. Pitz, B. Stoffelns, N. Pfeiffer, and S. Bhakdi. 2002. Extensive deposits of C3d and C5b-9 in the choriocapillaris of eyes of patients with diabetic retinopathy. *Invest. Opthalmol. Vis. Sci. 43:1104.*
39. Biesecker, G., and C. M. Gomez. 1989. Inhibition of acute passive transfer experimental auto-immune myasthenia gravis with Fab antibody to complement C6. *J. Immunol. 142:2654.*
40. Wang, Y., S. A. Rollins, J. A. Madri, and L. A. Matis. 1995. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl. Acad. Sci. USA 92:8955.*
41. Wang, Y., Q. Hu, J. A. Madri, S. A. Rollins, A. Chodera, and L. A. Matis. 1996. Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc. Natl. Acad. Sci. USA 93:8563.*
42. Amin, R., J. E. Puklin, and R. N. Frank. 1994. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. *Invest. Opthalmol. Vis. Sci. 35:3178.*
43. Ogata, N., M. Matsushima, Y. Takada, T. Tobe, K. Takahashi, X. Yi, C. Yamamoto, H. Yamada, and M. Uyama. 1996. Expression of basic fibroblast growth factor mRNA in developing choroidal neovascularization. *Curr. Eye Res. 15:1008.*
44. Matsushima, M., N. Ogata, Y. Takada, T. Tobe, H. Yamada, K. Takahashi, and M. Uyama. 1996. FGF receptor 1 expression in experimental choroidal neovascularization. *Jpn. J. Ophthalmol. 40:329.*
45. Ogata, N., C. Yamamoto, M. Miyashiro, H. Yamada, M. Matsushima, and M. Uyama. 1997. Expression of transforming growth factor-β mRNA in experimental choroidal neovascularization. *Curr. Eye Res. 16:9.*
46. Kwak, N., N. Okamoto, J. M. Wood, and P. A. Campochiaro. 2000. VEGF is stimulator in model of choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci. 41:3158.*
47. Krzystolik, M. G., M. A. Afshari, A. P. Adamis, J. Gaudreault, E. S. Gragoudas, N. A. Michaud, W. Li, E. Connolly, C. A. O'Neill, and J. W. Miller. 2002. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch. Ophthalmol. 120: 338.*
48. Johnson, L. V., S. Ozaki, M. K. Staples, P. A. Erickson, and D. H. Anderson. 2000. A potential role for immune complex pathogenesis in drusen formation. *Exp. Eye Res. 70:441.*
49. Mullins, R. F., S. R. Russell, D. H. Anderson, and G. S. Hageman. 2000. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *FASEB J. 14:835.*
50. Anderson, D. H., R. F. Mullins, G. S. Hageman, and L. V. Johnson. 2002. A role for local inflammation in the formation of drusen in the aging eye. *Am. J. Ophthalmol. 134:411.*
51. Ishibashi, T., T. Murata, M. Hangai, R. Nagai, S. Horiuchi, P. F. Lopez, D. R. Hinton, and S. J. Ryan. 1998. Advanced glycation end products in age-related macular degeneration. *Arch. Ophthalmol. 116:1629.*

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