Loss and Restoration of Immune Privilege in Eyes With Corneal Neovascularization

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Purpose. To delineate the time course for loss of immune privilege after induction of corneal neovascularization (NV), and to test whether treatment with angiostatic agents can restore the eye's capacity to induce anterior chamber-associated immune deviation (ACAID).

Methods. Corneal NV in murine eyes was induced by placement of intrastromal sutures. At different time points after NV induction, study eyes were initiated on a 10-day regimen of one of a variety of anti-inflammatory or angiostatic agents. After the completion of their treatment regimen, eyes were tested as to whether they could support ACAID. To test whether any observed effect on the delayed-type hypersensitivity response was because of a systemic absorption of the topically applied medication, certain animals had only their fellow eyes treated.

Results. Inflammatory corneal NV leads to the loss of immune privilege during the first week of the NV induction. Left untreated, these eyes remain incapable of supporting ACAID, even weeks after the initial corneal insult. However, when treatment with an anti-inflammatory agent is initiated during the first 2 weeks after the NV induction, these eyes show a restored capacity for ACAID induction, and this appears to be unrelated to any systemic effect of the treatment regimen. Treatment started at later time points is not capable of restoring the eye's normal capacity for inducing deviant immunity.

Conclusions. Corneal neovascularization leads to loss of immune privilege in the anterior segment manifested as the inability to sustain ACAID. Moreover, topical angiostatic strategies can lead to restoration of immune privilege when instituted sufficiently early in the course of the neovascular response. Invest Ophthalmol Vis Sci. 1996;37:2485-2494.

Corneal neovascularization (NV) is a ubiquitous element of corneal disease that can accompany an array of traumatic, inflammatory, infectious, toxic, and nutritional insults. Although it can sometimes be beneficial in the clearing of infections, wound healing, and in arresting progressive immune-mediated corneal melts, its disadvantages are more numerous and prevalent. Corneal NV frequently accompanies the most common causes of corneal infectious blindness in both the developed (herpetic keratitis) and underdeveloped (trachoma) worlds, and itself can lead to visual impairment by means of tissue scarring and lipid keratopathy.

In the setting of penetrating keratoplasty, neovascularization of the recipient corneal bed significantly increases the risk of graft rejection and ultimate failure. Furthermore, not only can antecedent corneal NV jeopardize allograft survival, but development of postpenetrating keratoplasty corneal NV also may pose risks to graft longevity. In fact, stratification of risk factors for immunologic rejections, including the degree of allos disparity, has shown recipient vascularization as the principal (i.e., clinically evident) cause of earlier and more fulminate rejection episodes. Whereas the success rate of corneal transplantation in low-risk settings surpasses 90%, the results are quite unsatisfactory when grafts are placed in high-risk neovascularized beds in which corneal grafts fare far worse than first allografts of kidney, heart, or liver. The exact reasons why corneal allografts fare so poorly in a setting of corneal NV are incompletely understood but are thought to be because of the loss of the normal immune privilege enjoyed by healthy

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Immune privilege in the eye is a complex entity that is best defined operationally. In its classic sense, it is defined as the extended (or even indefinite) survival of allografts placed in ocular compartments such as the anterior chamber (AC) or the vitreous cavity. Anterior chamber-associated immune deviation (ACAID) is defined as an antigen-specific systemic response evoked by intracameral injection of soluble antigens, characterized by a deficiency of delayed-type hypersensitivity (DTH)-mediating T cells and of complement-fixing antibodies. The stereotypical ACAID response is distinct from the conventional immune response, such as that seen with subcutaneous inoculations of antigen, in that it leads to a long-lasting systemic suppression of antigen-specific DTH. Immune privilege is thought to represent an evolutionary adaptation that is designed to limit intraocular expression of immunogenic inflammation, and in this context, it is thought that privilege depends, in large part, on the induction of ACAID.

In this series of experiments, we have therefore selected the capacity for ACAID induction to serve as our proxy measure of immune privilege.

It has been established that placement of intrastromal sutures induces an intense inflammatory neovascular response in the corneal stroma, rendering these eyes eventually incapable of supporting ACAID. This loss in the capacity to sustain ACAID has been correlated with the altered immunobiology of corneal allografts in high-risk settings where the grafts (unlike those placed in normal avascular beds) become targets of an intense donor-specific DTH response. In this series of experiments, we were interested to determine the time course over which immune privilege is lost subsequent to the induction of inflammatory corneal NV and to test whether topical treatment with angiostatic agents could restore the capacity to induce ACAID in eyes that had already lost that potential. By so doing, we wanted to delineate the window of opportunity during which therapeutic interventions may restore important features of immunogenic unresponsiveness (privilege). Furthermore, we wanted to determine if the hypothesized restoration of privilege in these treated neovascularized eyes could be explained by the possible systemic absorption of these therapeutic agents.

The results of our experiments clearly show that corneal NV leads to loss of immune privilege in the anterior segment within the first week after the neovascular response is induced, manifested by the inability to sustain ACAID. However, topical angiostatic strategies can lead to restoration of immune privilege when instituted sufficiently early in the course of the neovascular response, and the effectiveness of treatment does not appear to be directly related to either the degree of the NV at the time of initiating treatment or of intracameral injection. Furthermore, the restoration of ocular immune privilege appears to be related to the local immunomodulatory effects of the medications and not to their systemic absorption.

MATERIALS AND METHODS

Mice and Anesthesia

Six to 10-week-old BALB/c mice were purchased (Taconic, New York) or obtained from the Schepens Eye Research Institute's animal colony. All animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Each animal was anesthetized with an intramuscular injection of 3 to 4 mg ketamine and 0.1 mg xylazine.

Induction and Grading of Corneal Neovascularization

Three interrupted sutures (11-0 nylon, 50-μm needle, Sharpont; Vanguard, Houston, TX) were placed in the central cornea of one eye of a healthy BALB/c mouse under aseptic microsurgical technique using an operating microscope. As described previously, these sutures induce neovascular growth in the corneal stroma from the limbus that can be appreciated as early as 3 days after suture placement. Immediately after surgery, erythromycin ophthalmic ointment was applied, and the corneas were followed by slit-lamp biomicroscopy for corneal NV development. Neovascularization was graded between 0 and 3, with increments of 0.5, using a grid system per corneal quadrant based on the centripetal extent of the neovascular branches from the limbus. Scores for each quadrant then were summed to derive the NV index (range, 0 to 12) for each eye.

Pharmacologic Angiostatic Strategies

The following topical preparations were applied to selected mice twice daily for 10 days, at time points determined by the experimental protocol: (1) Potentiated angiostatic steroid: tetrahydrocortisol-S (1 mg/ml; Sigma, MO) was suspended in 1.5% methylcellulose vehicle and mixed with β-cyclodextrin tetradecasulfate to a final concentration of 1 mg/ml with a steroid:cyclodextrin (w/w) ratio of 1:0.5 as described previously. The solution was vortexed vigorously with sterile glass beads and stored at 4°C for 48 hours before application. (2) Nonsteroidal anti-inflammatory drug (NSAID) diclofenac sodium 0.1% ophthalmic solution (Voltaren; CIBA, Atlanta, GA). (3) Potent anti-inflammatory and angiostatic agent, prednisolone acetate 1.0% (Pred-Forte; Allergan, Irvine, CA) ophthalmic suspension. Control mice received either no treatment or vehicle alone.
Anterior Chamber-Associated Immune Deviation Induction and Delayed-type Hypersensitivity Assessment

At specific time points described below, anesthetized BALB/c mice received 50 μg of soluble antigen ovalbumin (Sigma Chemical, St. Louis, MO) per 3 μl volume of Hanks' balanced salt solution, into the AC of their test eyes as described previously.36 Seven days later, they received an immunizing 100-μg dose of ovalbumin (emulsified in complete Freund's adjuvant) for a total volume of 100 μl injected subcutaneously into the nape of the neck. Seven days after subcutaneous immunization, the mice received intradermal inoculations of antigen (200 μg/10 μl) into the pinna of their ear. The ear swelling response at 24 and 48 hours, as a measure of DTH, was assessed using a micrometer (Mitutoyo, MTI, Paramus, NJ). The investigator was masked as to which group of animals the ear measurements were being taken from.

RESULTS

Corneal Neovascular Responses to Topical Treatments

A central question in delineating the role of corneal inflammatory angiogenesis in abrogating immune privilege is whether the observed differences in the capacity to induce ACAID as a result of neovascular regression (angiostasis) are reflective of the degree of corneal NV at the time of treatment or of antigenic challenge per se.

The data (Fig. 1) show that in the murine corneal NV model used in our laboratory, the angiogenic response peaks around 2 weeks after NV induction and is sustainable for many weeks after induction. The NSAID diclofenac has a weaker, and more delayed, effect on angiostasis compared to steroidal agents, which cause up to 75% of NV regression, and there was no significant tissue difference between the selective angiostatic agent (tetrahydrocortisol-S-β-cyclodextrin tetradecasulfate [TCS-CD]) and the prednisolone acetate.

This model of suture-induced NV is associated with corneal inflammation that becomes evident clinically 2 days after suture placement, peaks at 2 weeks, and becomes biomicroscopically inapparent at 4 weeks. This course is reflected at the histopathologic level by a shift from an intense neutrophilic response early to a significantly milder inflammatory response later in the NV course characterized by a mixed neutrophil-mononuclear infiltrate. In this model of corneal neovascularization, the iris vessels become engorged at both the clinical and pathologic levels with associated AC flare (protein leakage); however, there is no significant inflammatory response in the ciliary body.

Loss of Immune Privilege—Anterior Chamber-Associated Immune Deviation

Because the advent of inflammatory corneal NV is associated with a number of untoward effects on the corneal microanatomy that lead, ultimately, to the abrogation of immune privilege and ACAID, it is important to document the time course of this loss of privilege. In this experiment (Fig. 2), corneal NV was induced on day 0. One group of animals (n = 5) received intracameral injection of antigen on that same day; two other groups (each n = 5) received injections in their AC 7 or 14 days later. ACAID controls received injections of antigen in virgin eyes with no NV. In each case, including positive controls that received no AC injections, animals were immunized subcutaneously 7 days after their AC injections, followed by ear challenges 1 week later.

We hypothesized that the shorter the duration between the induction of the corneal NV and the AC injections (ACAID priming), the greater the likelihood of observing ACAID. The DTH response in animals with NV induction 14 days earlier (mean ear swelling at 24 hours ± standard error, 68 ± 11 μm) is not statistically distinguishable from the DTH response seen in subcutaneously immunized positive controls (mean 83 ± 8 μm) and is significantly elevated compared to ACAID controls (mean 13 ± 4 μm). When the AC injection is performed 7 days after the NV induction (mean 48 ± 8 μm), similar results were obtained. These results formally show that in fact the anterior segment's immune privilege is lost early (during the first week) after NV induction.

Restoration of Immune Privilege in Neovascularized Eyes

As described in the introductory section, the main question that led us to conduct these experiments was whether the deleterious effect of corneal NV on the tissue parameters associated with immune privilege could be reversed, and whether this restoration of privilege could be defined operationally as resurrecting the eye's capacity for ACAID induction. In the next experiment, we tested the hypothesis that the abrogation of ACAID, which coincided with the advent of inflammatory NV, can be countered with specific angiostatic treatments applied topically. The experimental protocol has been summarized in Table 1. Briefly, starting at 1 week after the induction of the corneal NV, test animals received twice-daily administrations of angiostatic agents as outlined in the Materials and Methods section. Ten days into this treatment, all animals received intracameral injections of antigen and were subsequently immunized subcutaneously and challenged dermally as outlined. The ACAID controls received AC inoculations of antigen in their virgin

FIGURE 1. Induction of corneal neovascularization in murine eyes. Groups of mice (n = 5) received topical angiostatic agents starting on day 7. Results of a representative experiment are shown. Agents (detailed in the Materials and Methods section) included diclofenac 0.1% (NSAID); prednisolone 1% (pred 1%); and tetrahydrocortisol-S-β-cyclodextrin tetradecasulfate (TCS-CD).

FIGURE 2. Capacity for anterior chamber associated immune deviation (ACAID) induction, measured as the ability to show delayed-type hypersensitivity downregulation after antigenic challenge, depending on the timing (number of days after induction of neovascularization) of intracameral antigen inoculation. The ACAID controls received inoculations in non-neovascularized eyes; positive controls did not receive any intracameral antigen. The ACAID induction capacity is lost within 1 week of inciting corneal NV.
TABLE 1. Protocol for Testing the Effect Treatment With Angiostatic Agents on the Capacity of the Neovascularized Eye to Sustain ACAID

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Days</th>
<th>Day 17</th>
<th>Day 24</th>
<th>Day 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Virgin/positive control</td>
<td>None</td>
<td>—</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
<tr>
<td>2. Virgin/ACAID control</td>
<td>None</td>
<td>+</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
<tr>
<td>3. NV, control</td>
<td>Placebo</td>
<td>+</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
<tr>
<td>4. NV, test group 1</td>
<td>Prednisolone acetate 1%</td>
<td>+</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
<tr>
<td>5. NV, test group 2</td>
<td>Tetrahydrocortisol-S-CD</td>
<td>+</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
<tr>
<td>6. NV, test group 3</td>
<td>NSAID, diclofenac 0.1%</td>
<td>+</td>
<td>Subcutaneous</td>
<td>+</td>
</tr>
</tbody>
</table>

ACAID = anterior chamber-associated immune deviation; AC = anterior chamber; NV = neovascularized; NSAID = nonsteroidal anti-inflammatory drug; CD = cyclodextrin.

(i.e., avascular) eyes, and positive controls received no AC inoculations but were immunized subcutaneously before intrapinna antigenic challenge.

The results of the DTH responses 24 hours after antigenic challenge (similar results at 48 hours; data not shown) are summarized in Figure 3. The data show conclusively that treatment for 10 days with any of the three angiostatic agents restored the ACAID response. Whereas the DTH (mean ear swelling ± standard error) response in the untreated NV control group (69 ± 13 μm) was not significantly different from the response in the positive controls (102 ± 11 μm), all three NV groups that had undergone angiostatic treatment had significantly suppressed DTH responses (range, 15 to 18 μm) that were indistinguishable from that observed in the ACAID control group (24 ± 6 μm). Furthermore, to our surprise, there was no appreciable difference in the degree of DTH suppression achieved by different types of angiostatic agents. In companion experiments, we also tested the effect of a 10-day course of topical steroidal treatment with prednisolone acetate 1% on the ACAID response in virgin eyes and detected no appreciable difference as compared to untreated ACAID controls or naive animals (data not shown).

**Effect of Angiostatic Treatment Timing on Capacity to Restore Immune Privilege**

We proceeded to evaluate the effect of the timing of our angiostatic interventions on immune privilege.
Specifically, our goal was to test whether the same treatment protocol, which when initiated 1 week after induction of inflammatory NV and continued for 10 days, had led to the restoration of the ACAID response could have a similar effect when started at later time points in the neovascular response. Because, in the previous set of experiments we had not seen any appreciable difference in the degree to which DTH was blunted based on the specific pharmaceutical used (Fig. 3), we selected the most potent anti-inflammatory agent (1% prednisolone acetate) for these series of experiments.

Accordingly, test animals were treated with topical prednisolone twice a day for 10 days; test groups differed in the time points at which they were initiated on their therapeutic regimens. Similar to the other DTH assays described above, animals received intracameral inoculations of antigen after completing their topical therapy and were subsequently immunized subcutaneously 1 week later and ear challenged 1 week after that. The results of this series of experiments (Fig. 4) showed that the corneas that had undergone treatment during days 14 to 24 had a blunted DTH response (mean ear swelling ± standard error, 21 ± 3 μm) that was statistically not significantly different from the DTH response seen in ACAID controls (11 ± 3 μm). In contrast, the DTH responses in mice whose NV corneas had been treated during days 21 to 31 (57 ± 4 μm) or days 28 to 38 (62 ± 13 μm) were indistinguishable from the response in positive controls (71 ± 5 μm). These data established that the ability to restore ACAID to neovascularized eyes is dependent on when the corneal is treated after initiation of the NV response.

**Systemic Versus Local Effect of the Therapeutic Agent**

We next wished to differentiate between a local effect of the anti-inflammatory treatment on ocular immune privilege and a systemic effect brought about via systemic absorption of the pharmaceuticals. We hypothesized that the systemic absorption of the topically applied agents was responsible for the DTH downregulatory response we had observed as a result of the intracameral inoculations of antigen in the treated NV eyes.

Animals were categorized into five groups; all (except the ACAID and positive controls) with corneal NV in their index eyes (Fig. 5). Seven days after induction of NV, two groups received topical angiostatic treatment with 1% prednisolone in their fellow virgin or NV eyes, with no treatment in their index eyes. Ten days into this treatment regimen, the index eyes received intracameral inoculations of antigen after which the mice received subcutaneous immunizations and intradermal antigen challenges as described above. The positive controls received no AC inoculation, and the standard ACAID controls received AC inoculations in a virgin eye only. As an additional control, a last group (designated NV index Tx in Fig. 5) received angiostatic treatment in the neovascularized index eye only.

The results show that there is no statistically significant difference in the DTH responses among animals that had received treatment in their fellow eyes (regardless of the NV status in those eyes) and the positive controls (Fig. 5). This strongly suggests that systemic absorption of the treatment regimens (if any) is not the determining factor in the restoration of an
Immune Privilege and Corneal Neovascularization

FIGURE 5. Delayed-type hypersensitivity responses to antigen in animals with neovascularized (NV) eyes whose fellow virgin eye (virgin fellow Tx) or fellow NV eye (NV fellow Tx) received a 10-day course of angiostatic treatment before receiving intracameral antigen inoculation in the index NV eye. Controls included animals that received the same treatment in the index eye only (NV index Tx), positive controls that received no intracameral inoculation, and anterior chamber associated immune deviation controls that received inoculations in their avascular index eyes. Delayed-type hypersensitivity suppression is seen only when the index (antigen-inoculated) eye is treated.

ACAID-type response in neovascular index eyes. We conclude that the effect of these angiostatic agents on the capacity of the treated eyes to sustain ACAID is a local immunomodulatory one.

DISCUSSION

The loss of immune privilege in the eye likely has significant pathophysiologic implications, even in eyes that are not transplant recipients. The dysregulatory immunogenic mechanisms that are set in motion as a result of the eye’s loss of its normal immune privileged state can pose a threat to sight because the eye can become a target of immunogenic inflammation in ways not too dissimilar from the way inflammation can affect other (nonprivileged) sites.16-18,19,27

Corneal NV is an important pathogenic mechanism for inciting, and maintaining, an abnormal microenvironmental milieu that is inhospitable to the anterior segment’s normal immune privileged and quiescent state.16,17,25 As such, many corneas suffering from clinically significant NV not only opacity, but render the corneal bed a high-risk site for engraftment.7,8,15 In fact, the level of risk posed by corneal NV is so significant that many of the patients suffering from it are never considered as serious candidates for corneal transplantation because of the surgeons’ a priori sense that the allografts are doomed to fail. Conversely, the extraordinary success of corneal allografts has been attributed to various features of corneal microanatomy and physiology, which, in the aggregate, account for the immune privileged status of the anterior segment.14,16

The results of this series of experiments, in addition to other work in our laboratory, suggest that cardinal features of the anterior segment that are critical to the maintenance of immune privilege may be disrupted in corneal inflammation. These features include the avascularity of the cornea that stringently limits the ingress of immune and inflammatory mediators in to the tissues of the anterior segment, the absence of corneal lymphatics, the rarity of conventional antigen-presenting cells, and a unique spectrum of cornea- and iris-ciliary body-derived immunomodulatory cytokines that suppress immunogenic inflammation and complement activation.10,21-25 Recently, the expression of Fas ligand by certain ocular cells has been related to the capacity of the eye to maintain its immune privileged state28; however, we have not explored this parameter in relation to corneal neovascularization.

Our goal in the present study was to explore the interface between immune privilege and corneal NV, specifically to answer the basic question of whether the loss of immune privilege can be reversed by local treatments that can cause subtotal regression of the
NV. In the aggregate, the results of these experiments indicate the following:

1. The immune privilege present in the anterior segment is lost within the first week of corneal NV induction (Fig. 2).
2. Treatment with a variety of anti-inflammatory and angiostatic agents can cause significant (but never absolute) regression of the NV response and can restore ACAID to eyes that have lost privilege if the treatment is initiated sufficiently early (Figs. 3, 4).
3. The immunomodulatory effect of these therapeutic agents is manifest locally rather than systemically (Fig. 5).

The relative contributions of the local factors responsible for the restoration of privilege are understood incompletely. First, it is possible that regression of the corneal angiogenesis leads to a significant decrease in the blood-ocular barrier breakdown, thereby reducing the activity of immune effectors (or alternatively upregulating the activity of constitutive immune suppressants) in the anterior segment. For example, leakage of normal plasma constituents such as α2-macroglobulin or proteases from corneal neovessels into the cornea and anterior segment could neutralize the activity of transforming growth factor-β and neuropeptides (e.g., α-melanocyte-stimulating hormone) that have been implicated in ocular immune privilege. Furthermore, in the uninflamed eye, there is little binding of cortisol in the aqueous humor by globulins, a possibly critical feature of the microenvironment in the healthy eye. This situation can be reversed readily by breakdown of the blood-aqueous barrier secondary to the ocular inflammatory response. However, any reversal in the degree to which this functional barrier is perturbed in inflammation may tilt the balance (once again) in favor of the eye's normally privileged state where both the afferent and efferent arcs of immunity favor a state of unresponsiveness.

Second, it also is possible that the restoration of ACAID is dependent on other key parameters that define immune privilege, such as the afferent arc of immunity, including the degree of lymphatics or activity of antigen-presenting cells (APC). Corneal lymphangiogenesis has been described morphologically as accompanying inflammatory corneal NV. In addition, we have collected data recently (not presented herein) from both immunohistochemical as well as functional studies in the mouse that show clearly the development of lymphatic outflow from neovascularized corneas. These channels can serve as efficient conduits of (whole or APC-processed) antigen fragments to the highly immunogenic milieu of the draining lymph nodes. We also have accumulated preliminary data that suggest that the angiostatic protocols used in this series of experiments also blunt the degree of lymphatic outflow from the anterior segment.

Finally, the anti-inflammatory strategies (e.g., application of prednisolone) that lead to angiostasis by their wide-ranging effect on inflammatory cellular and molecular effectors also can cause a downregulation in the number and activity profiles of professional APCs, thereby having a dampening effect on these critical participants of the immune response. Because an increase in the number of indigenous APCs (Langerhans cells) in the central and paracentral cornea invariably accompanies inflammatory NV, particularly in the early stages of the process, downregulation in the number of these cells as a result of the therapeutic interventions likely facilitates restoration of privilege by reversing antigen processing facility afforded by the increased presence of these cells. We hypothesize that injected antigen can gain access to the APCs, directly within the milieu of the cornea-anterior segment or indirectly by blackflow through the needle track. Additionally, the edematous corneal stroma in the inflammatory model of NV may have altered permeability characteristics that can facilitate both antigen diffusion as well as dendritic cell migratory capacity. It has been shown that a few hapten-derivatized Langerhans cells are capable of mediating contact hypersensitivity. A similar mechanism may be at work in the neovascularized eye, so that even a small number of APCs can effectively sensitize the animal by delivering processed antigen to the immunogenic milieu of the node via lymphatics. However, we cannot ascertain, based on these experiments, if the angiostatic treatment itself had the capacity to induce a direct ACAID-inducing signal by affecting the newly recruited APCs in the anterior segment.

From a therapeutic standpoint, in our model of inflammatory corneal NV, the window of opportunity for restoring immune privilege is quite narrow. Regimens that result in significant degrees of angiostasis, but are delayed in onset, do not have an appreciable effect on the eye's capacity to promote deviant immunity. If left untreated, ACAID is lost in the first week after NV induction, a significantly earlier time point than when maximal NV is reached.

A logical question would be whether the effect of the treatment on restoration of immune privilege is dependent on the degree of corneal NV. Although we cannot offer a definitive response in relation to the question of "threshold" angiogenesis required to abrogate ACAID, we can state that the data formally offer proof that the degree of clinically evident corneal NV is not responsible directly for the dysregulatory immunologic responses that we observed in inflamed eyes. We can review the evidence two ways: by examining the degrees of NV at the time of initiating treatment.
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**References**


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