

Effect of Allergic Conjunctival Inflammation on the Allogeneic Response to Donor Cornea

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PURPOSE. Immunologic rejection is the most common cause of corneal allograft rejection. Ipsilateral ocular inflammation has been identified as a predictor of future corneal graft failure. This study investigates the effect of perioperative allergic conjunctivitis on corneal allograft survival.

METHODS. C57BL/6 donor corneas were transplanted into naive A/J mice, A/J mice sensitized to short ragweed (SRW) pollen by intraperitoneal injection and then challenged with topical SRW to induce allergic conjunctivitis (Sens⁺Chall⁺), and A/J mice sensitized to SRW and challenged with topical PBS (Sens⁺Chall⁻). Syngeneic grafts were also performed in eyes with allergic conjunctivitis. Graft survival and infiltrating cell phenotype in rejected grafts were compared between groups.

RESULTS. Mice with allergic conjunctivitis (Sens⁺Chall⁺) rejected corneal allografts significantly more quickly than naive mice. Syngeneic grafts in allergic eyes survived indefinitely. The rate of rejection in Sens⁺Chall⁻ mice was similar to that in naive mice. There were no significant differences, between groups, in the numbers of infiltrating CD4⁺ cells, CD8⁺ cells, and macrophages at the time of graft rejection. Eosinophils were seldom observed in rejected grafts in naive and Sens⁺Chall⁻ mice but were observed consistently in Sens⁺Chall⁺ eyes. Eosinophils were also found consistently in the ciliary body of Sens⁺Chall⁺ eyes at the time of graft rejection.

CONCLUSIONS. Active allergic conjunctivitis at the time of transplantation accelerates corneal allograft rejection. Local conjunctival inflammation is an important factor in accelerating rejection. (*Invest Ophthalmol Vis Sci.* 2007;48:4044–4049) DOI:10.1167/iovs.06-0973

More than 100 years after the first human corneal allograft, many of the factors likely to lead to graft failure have been identified. Unfortunately, the prognosis for survival in these “high-risk” grafts has improved little in that time. Corneal vascularization, previous graft failure, and glaucoma are all associated with an accelerated rate of graft failure, usually because of immune-mediated rejection. Ipsilateral ocular inflammation has also been identified as a predictor of graft

failure.¹ Furthermore, the timing of this inflammation appears to be important, with perioperative inflammation signifying the worst prognosis.

The aim of this study was to investigate the effect of a specific type of perioperative ocular inflammation, allergic conjunctivitis, on corneal allograft rejection. Allergic conjunctivitis is important in the context of corneal transplantation for 2 reasons. First, it is the most prevalent form of ocular inflammation in general.² It may actually be overrepresented in corneal transplant patients given the association between allergic eye disease and keratoconus,^{3,4} the usual indication for corneal transplantation.^{5,6} Second, atopy is associated with a skewing of the T-helper cell immune responses toward Th2.^{7,8} Alterations in Th1/Th2 bias may influence the immune response to an allograft.

Convergent studies have identified the CD4 (T-helper [Th]) cell as the key effector cell in corneal allograft rejection.^{9,10} Activated Th cells secrete cytokines, which in turn activate and recruit effector cells. Th cells may be classified as Th1 (IL-2, IFN- γ) or Th2 (IL-4, IL-5, IL-10), depending on the profile of their cytokine secretion. Traditionally, allograft rejection has been thought to be a Th1-mediated process.¹¹ This is largely true of unmodified corneal transplantation.^{12,13} However, Th2 and Th1 cells cross-regulate each other. It has been hypothesized that by enhancing the Th2 response, the Th1 response would be attenuated and graft tolerance would be achieved.¹⁴ Experimental strategies to deviate the immune response toward Th2 in cardiac allografts have had mixed results in terms of allograft survival.^{15–17} However, one thing has become clear: a Th2-dominant response to alloantigen is capable of graft destruction, possibly via novel effector mechanisms such as eosinophilic infiltration.¹⁸

Prior sensitization to allergen has been shown to induce an increased Th2 response to *alloantigen*. As in other types of allograft, the effects of this on corneal allograft survival have been mixed. In a model of high-risk corneal transplantation to a vascularized recipient bed, Th2 bias improved graft survival.¹⁹ However, in a model of normal risk transplantation, accelerated corneal allograft rejection was found in patients with allergic conjunctivitis, and this was attributed to the Th2 bias induced by systemic sensitization with allergen.²⁰ Our study examines the effect of perioperative allergic ocular inflammation on allograft survival and on the composition of the inflammatory infiltrate during rejection.

METHODS

Animals

Female 6- to 8-week-old A/J strain mice (H-2^b; Harlan UK, Bicester, UK) were used in the allergic conjunctivitis induction protocol and subsequently as corneal allograft recipients. Adult female C57/BL6 strain (H-2^b; Harlan UK), which provide a full major histocompatibility complex (MHC) mismatch and multiple minor mismatches, were used as graft donors. An additional control group of A/J mice received syngeneic grafts (Table 1). Animals were treated in accordance with the UK government regulations for the care of experimental animals and with

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TABLE 1. Experimental Groups for Transplantation

Recipient	Graft	Sensitized	Challenged
Naive	Allograft	No	No
Isograft	Isograft	Yes	Yes
Sens ⁺ Chall ⁺	Allograft	Yes	Yes
Sens ⁺ Chall ⁻	Allograft	Yes	No
Sens ⁻ Chall ⁺	Allograft	No	Yes

the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research

Induction of Allergic Conjunctivitis

Mice were sensitized to the allergen short ragweed (SRW) pollen over a 15-day period using a previously described method.²¹ This sensitization period is required for the generation of systemic Th2 responses, and subsequent challenge with topical SRW results in severe allergic conjunctivitis. Mice were sensitized by intraperitoneal injection of SRW (Greer Laboratories, Inc., Lenoir, NC) pollen, 200 μ g with 2 mg aluminum hydroxide (Alum; Sigma, St. Louis, MO) in 0.4 mL phosphate-buffered saline (PBS) on days 0, 7, and 14. The sensitization period also involved treatment with eyedrops (SRW pollen 500 μ g with 25 μ g aluminum hydroxide [Alum; Sigma] in 5 μ L PBS) on days 8 and 15. The experimental challenge of 500 μ g in 5 μ L PBS was administered topically to the right eye on day 27, immediately after corneal transplantation, for the sensitized and challenged (Sens⁺Chall⁺) group. PBS 5 μ L without SRW pollen was instilled after corneal transplantation in the sensitized-non challenged (Sens⁺Chall⁻) group.

Induction of Corneal Inflammation

To induce corneal inflammation, four 11.0 nylon sutures were placed in the paracentral corneal stroma of A/J mice and were removed after 1 week.

Transplantation Technique and Diagnosis of Rejection

The technique for corneal transplantation was as previously reported.²² Mice were anesthetized with intraperitoneal fentanyl flunitrazepam and midazolam. All grafts were performed in the right eye. A 2.5-mm donor button was sutured into a 2.0-mm recipient corneal bed with a continuous 11-0 nylon suture. At the end of the procedure, tarsorrhaphy was performed. This was opened after 48 hours; eyes with infection, hemorrhage or cataract were excluded. Thereafter, the eyes were examined three times weekly under brief inhalational isoflurane anesthesia, and the graft graded as follows: 0 = completely transparent cornea; 1 = minimal corneal opacity, but iris vessels easily visible; 2 = moderate corneal opacity, iris vessels still visible; 3 = moderate corneal opacity, only pupil margin is visible; 4 = complete corneal opacity, pupil not visible.

Corneal sutures were removed at 7 days. Corneal graft rejection was diagnosed when the corneal clarity score increased to 3 in a graft previously transparent after surgery.

Immunohistochemistry

On diagnosis of rejection, mice were killed and the whole eye was enucleated. The eye was embedded in optimal cutting temperature compound (OCT; Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and was frozen on a liquid nitrogen-cooled duralumin plate. Specimens were stored at -70°C . Cryostat sections 8- μ m thick were cut, and indirect frozen section immunohistochemical analysis was performed using the following primary antibodies: rat anti-mouse CD4, RM4-5 (BD Biosciences, San Jose, CA) 1:100 dilution; rat anti-mouse CD8, YTS105.18 (Serotec, Raleigh, NC) 1:100 dilution; rat anti-mouse F4/80, Cl:A3-1 (Serotec) 1:300 dilution; and rat anti-mouse major basic protein (kind gift from Dr. J. Lee, Mayo Clinic,

Scottsdale, AZ). Mouse anti-rat IgG₁, IgG_{2a} and IgG_{2b} isotype controls (all from Serotec) were used at appropriate dilutions as controls. Positive-staining cells in the central cornea and the ciliary body were counted. Because rejected corneal allografts demonstrate variable thickness resulting from edema, it was not appropriate to count the number of cells per unit area. Instead, the number of positive cells throughout the full thickness of a 100 $\times$ field of the central stroma of each section was counted. Cells were counted in three sections per rejected graft. At least five grafts were examined in each group. Sections of the ciliary body were imaged, and their cross-sectional areas were measured using image analysis software (Soft Imaging System GmbH, Munster, Germany). The number of positive-staining cells in each ciliary body section was counted using high magnification and expressed as cells/0.1 mm². Cells were counted in three sections per eye. At least five eyes were examined in each group.

To study the effects of the sensitization protocol (without challenge) on the recipient cornea, we compared frozen sections from Sens⁺Chall⁻ eyes with normal eyes and with eyes with suture-induced corneal inflammation. Sections were stained directly with phycoerythrin (PE)-conjugated rat anti-mouse CD11b, M1/70 (BD Biosciences), dilution 1:100, and PE-conjugated rat anti-mouse CD11c, B-ly6 (BD Biosciences), dilution 1:100. Other sections were stained with primary rat anti-mouse LYVE-1, 223322 (R&D Systems Minneapolis, MN), dilution 1:400, and then with secondary Alexa-488-conjugated donkey anti-rat antibody (Invitrogen, Carlsbad, CA). Four eyes per group were examined.

Statistical Analysis

Median graft survival time (MST) was calculated for each group, and Kaplan-Meier survival curves were constructed.²³ Survival was compared using the log-rank test. The unpaired Student's *t*-test was used to compare numbers of graft-infiltrating leukocytes between groups. The χ^2 test was used to compare groups for the presence of infiltrating eosinophils. For each statistical test, *P* < 0.05 was defined as statistically significant.

RESULTS

Corneal Immunohistochemistry after Sensitization

Large numbers of infiltrating CD11b⁺ and CD11c⁺ cells were seen in the cornea after placement of corneal sutures. LYVE-1 staining was also consistently seen in the corneal stroma in these eyes. By comparison, few CD11b⁺ and CD11c⁺ cells and no LYVE-1 staining were seen in normal and in Sens⁺Chall⁻ corneas (Fig. 1).

Survival of Corneal Allografts

Baseline survival of C57BL/6 allografts in naive A/J mice was first established. By 60 days, 73% of these naive A/J mice rejected their allografts (MST, 36 days). Allografts were then placed in A/J mice that had been sensitized to SRW pollen. Immediately after transplantation, these sensitized mice were challenged with SRW eyedrops in the transplant recipient eye to induce allergic conjunctivitis. All (100%) these sensitized and challenged (Sens⁺Chall⁺) A/J mice rejected their allografts at a significantly lower MST of 16 days (*P* < 0.001). Next, corneal allografting was performed in sensitized mice that were then mock-challenged with PBS in the graft recipient eye. These mice, which were sensitized but not challenged (Sens⁺Chall⁻), rejected their grafts at a rate similar to that of naive mice, with 71% of grafts rejected at an MST of 32 days (Fig. 2). The rate of rejection of these grafts was significantly slower than that in Sens⁺Chall⁺ mice (*P* = 0.001).

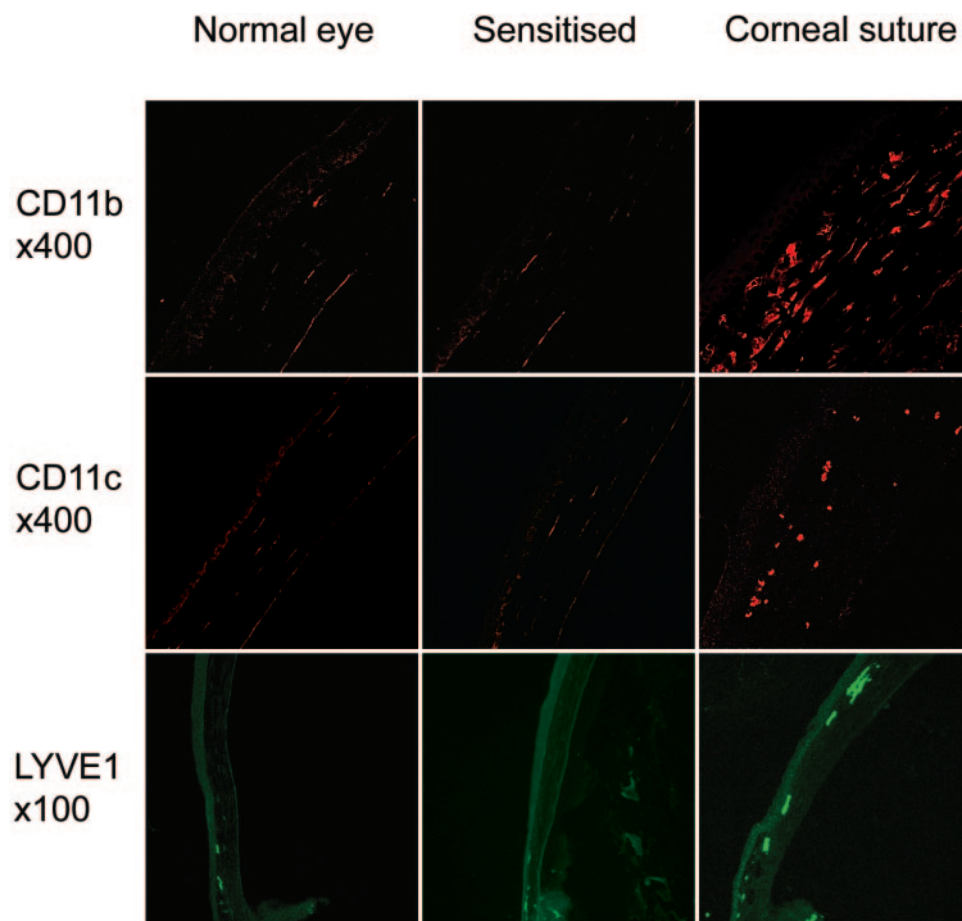


FIGURE 1. Corneal APCs and lymphatics. Although the sensitization protocol did involve topical exposure to SRW, it did not alter the corneal stromal content of dendritic cells (CD11c) or macrophages (CD11b) and did not induce the formation of new lymphatics (LYVE-1). This finding is in contrast to the established model of high-risk corneal transplantation (suture-induced corneal inflammation).

All grafts in Sens⁻Chall⁺ eyes were rejected, but the rate of rejection was significantly slower than that in Sens⁺Chall⁺ eyes and was not significantly different from that in naive eyes.

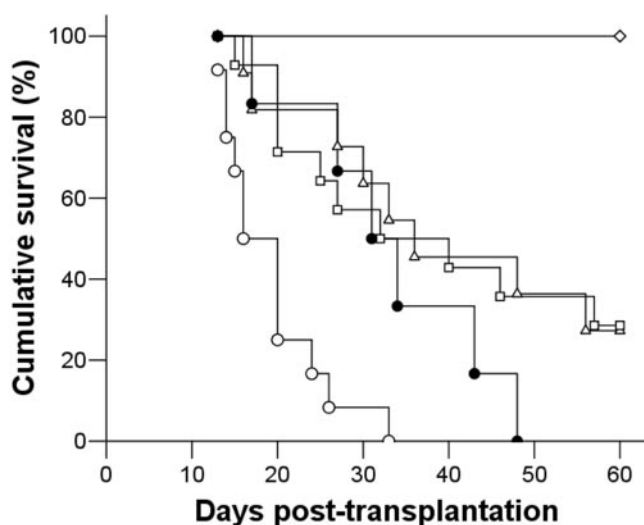


FIGURE 2. Actuarial corneal transplant survival. Allografts in Sens⁺Chall⁺ eyes (○; *n* = 12; MST = 16 days) were rejected at a significantly faster rate than those in naive eyes (△; *n* = 11; MST = 36 days), those in Sens⁺Chall⁻ eyes (□; *n* = 14; MST = 32), or those in Sens⁻Chall⁺ eyes (●; *n* = 6; MST = 31 days). Isografts in sensitized and challenged eyes (◇; *n* = 4) survived beyond 60 days. *P* < 0.001 (○ vs. △; *P* = 0.001 (○ vs. □; *P* = 0.006 (○ vs. ●).

Survival of Isografts in Mice with Allergic Conjunctivitis

To establish whether graft failure in allergic mice was primarily caused by a specific response against alloantigens or nonspecific allergic inflammation, syngeneic corneal grafts were placed in sensitized A/J mice that were then challenged with SRW eyedrops in the graft eye to induce allergic conjunctivitis. All (100%) these syngeneic grafts survived for 60 days.

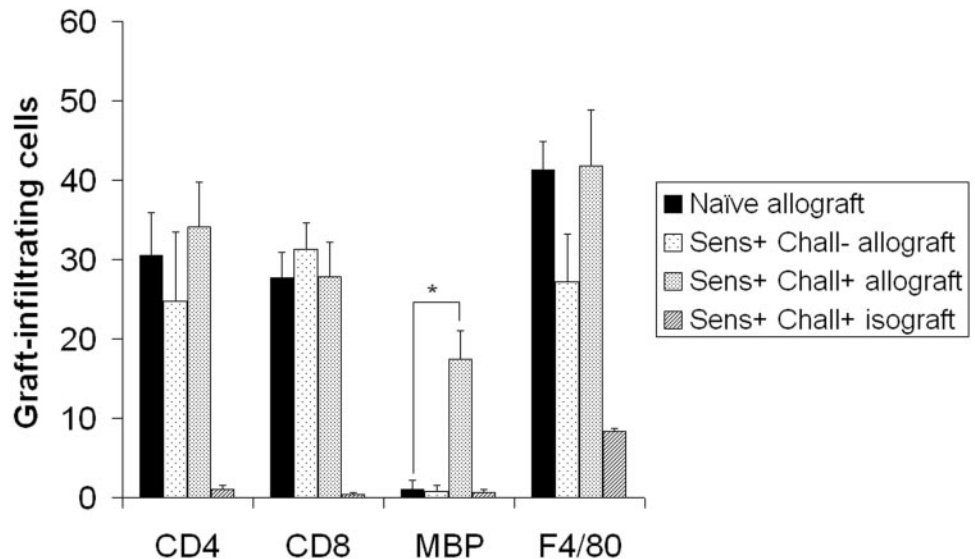
Composition of Graft Infiltrate during Acute Rejection

Phenotypes of graft-infiltrating cells were characterized using immunohistochemistry, and comparisons were made between rejected grafts in naive, Sens⁺Chall⁺, and Sens⁺Chall⁻ mice. No significant differences were observed in the numbers of CD4⁺, CD8⁺, and F4/80⁺ cells infiltrating the grafts at the time of rejection in all groups (Fig. 3). Within each group, no significant differences were observed in the numbers of graft-infiltrating CD4⁺, CD8⁺, and F4/80⁺ cells.

MBP⁺ cells were found consistently in rejected grafts in Sens⁺Chall⁺ mice but were seldom found in rejected grafts in naive mice or Sens⁺Chall⁻ mice (Fig. 4). Despite the significant association (*P* = 0.01) between the presence of perioperative allergic conjunctivitis and the presence of an eosinophilic infiltrate at the time of graft rejection, the number of graft-infiltrating eosinophils in Sens⁺Chall⁺ eyes was significantly lower than in CD4⁺ cells, CD8⁺ cells, or macrophages (Fig. 3).

Eosinophils were also seen consistently in the uveal tract of Sens⁺Chall⁺ eyes at the time of rejection (*P* = 0.003). They

FIGURE 3. Comparison of numbers of graft-infiltrating cells at the time of graft rejection. The number of cells in a 100 $\times$ field staining for CD4⁺, CD8⁺, MBP (eosinophils), and F4/80 (macrophages) were counted; mean $\pm$ SE values are shown. CD4 cells, CD8 cells, and macrophages were seen consistently in all allograft groups. Eosinophils were seen predominantly in allografts in eyes that had perioperative allergic conjunctivitis but not in isografts. * $P < 0.01$.



were not found in rejected grafts in Sens⁺Chall⁻ eyes and were seldom seen in naïve eyes (Fig. 5).

At the time of rejection, eosinophils were seen in the conjunctiva of Sens⁺Chall⁺ eyes but were not seen in Sens⁺Chall⁻ or naïve eyes. Eosinophils were also seen, at 60 days, in the conjunctiva of Sens⁺Chall⁺ eyes that had received isografts (Fig. 6). However, no eosinophils were seen in the isografts in Sens⁺Chall⁺ eyes at 60 days, nor were they seen in the ciliary body of these eyes. An additional group of isografts was placed in Sens⁺Chall⁺ eyes, and these eyes were removed for immunohistochemistry on postoperative day 20. In this group, eosinophils were found in the conjunctiva, but none were seen infiltrating the graft or ciliary body (data not shown).

DISCUSSION

Corneal graft outcome data from large cohort studies have demonstrated the negative impact on survival of ocular inflammation at the time of transplantation.^{2,4} The results of our study are consistent with these data and show that allergic conjunc-

tivitis, in particular, accelerates corneal allograft rejection. This result is also consistent with the finding by Beauregard et al.²⁰ of an increased rate of corneal graft rejection in their model of chronic postoperative allergic conjunctivitis. What we have

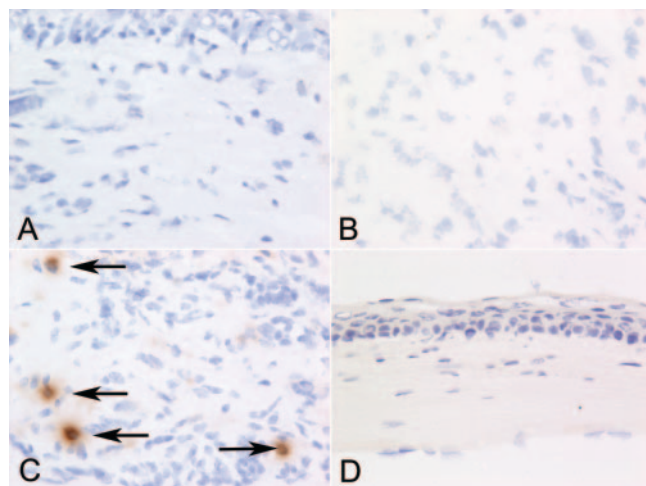


FIGURE 4. Immunohistochemical staining for major basic protein in corneal grafts. Eosinophils (arrows) were not seen in naïve allografts (A), Sens⁺Chall⁻ allografts (B) or Sens⁺Chall⁺ isografts (D) but were seen in Sens⁺Chall⁺ allografts (C). The stroma of the rejected allografts was thicker than in isografts.

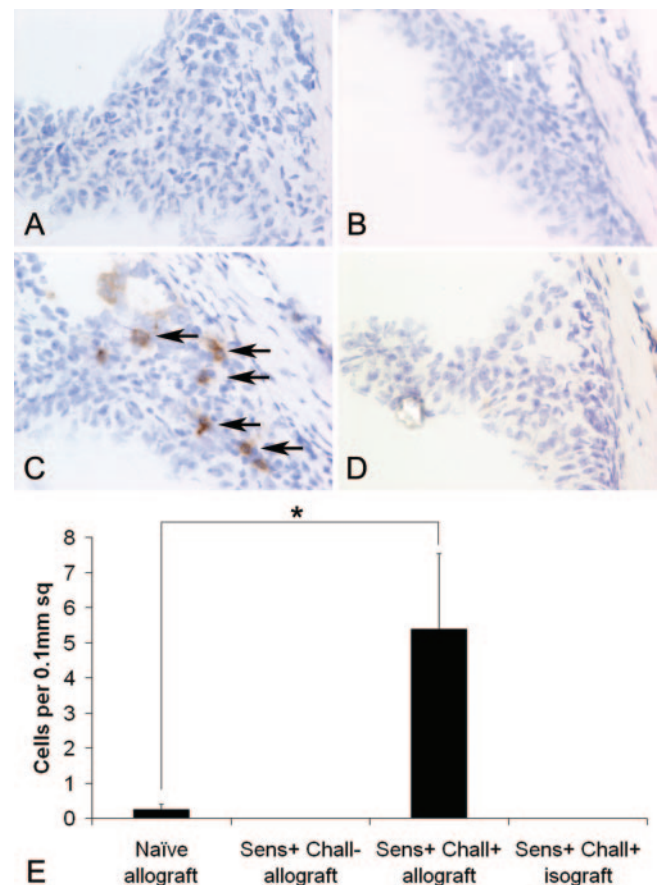


FIGURE 5. Immunohistochemical staining for major basic protein in ciliary body. Eosinophils (arrows) were rarely seen in naïve eyes (A) and were not seen in Sens⁺Chall⁻ eyes (B) but were seen in Sens⁺Chall⁺ eyes (C). No eosinophils were seen in the ciliary body of Sens⁺Chall⁺ recipients of isografts (D). The histogram (E) depicts the number of MBP-positive cells/0.1 mm² cells of ciliary body. * $P < 0.05$.

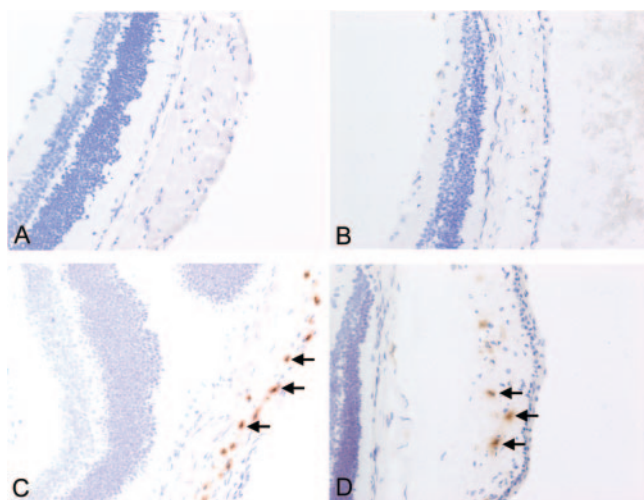


FIGURE 6. Immunohistochemical staining for major basic protein in bulbar conjunctiva. At the time of allograft rejection, eosinophils (arrows) were not seen in naive eyes (A) or Sens⁺Chall⁻ eyes (B) but were seen in Sens⁺Chall⁺ eyes (C). Eosinophils were also seen in the bulbar conjunctiva of Sens⁺Chall⁺ eyes 60 days after isograft (D).

shown is that allergic inflammation in the perioperative period alone is sufficient to shorten graft survival.

Animals in the model of allergic conjunctivitis we report underwent two interventions, either of which could in theory have influenced graft survival. The preliminary sensitization of animals to SRW has been shown by others to skew the immune response toward Th2.^{19,20} Subsequent challenge with topical SRW induces local ocular inflammation. It is of interest that animals sensitized to SRW but not challenged rejected allografts at a rate similar to that of naive animals. This is consistent with our finding that, after sensitization, the cornea appears normal in terms of its antigen-presenting cell (APC) and lymphatic content. Taken together, these findings suggest that local perioperative inflammation rather than any systemic effect of SRW sensitization is responsible for the increased rate of allograft rejection after perioperative allergic conjunctivitis. In other words, the presence of local perioperative inflammation confers the status of high rejection risk on the graft.

For reasons that are unclear, this finding is in contrast with the findings of Beauregard et al.,²⁰ who report that, in their model of allergic conjunctivitis, the increased rate of allograft rejection was attributable to increased Th2 responses rather than to local inflammation. Differences in the models of allergic conjunctivitis may in part explain the variance in results: a different strain of graft recipient mouse (A/J as opposed to BALB/c) was used in the experiments we report, and the allergic conjunctivitis induction protocol differed in that we challenged the conjunctiva with SRW only once rather than throughout the posttransplantation period of observation. On one hand, the survival results in animals with active allergic conjunctivitis (Sens⁺Chall⁺) were similar in both studies. The difference lies in the groups of animals sensitized but not challenged (Sens⁺Chall⁻), and here the protocols also differed. We designed Sens⁺Chall⁺ and Sens⁺Chall⁻ groups to represent, as closely as possible, the clinical picture seen in patients with allergic conjunctivitis and without active or uncontrolled disease. Therefore, our Sens⁺Chall⁻ animals received one mock challenge with PBS in the corneal graft (ipsilateral) eye and nothing in the contralateral eye. Graft recipients in the study by Beauregard et al.²⁰ received repeated mock challenges with PBS in the ipsilateral eye and repeated SRW challenges in the contralateral eye. It is not clear why SRW challenge in the contralateral eye should lead to accelerated graft

rejection. One possibility is that as mice rub their eyes vigorously after challenge with SRW, inadvertent contralateral transfer of SRW occurs. This also raises the possibility that accelerated graft rejection in these models of allergic conjunctivitis may result from the mechanical effects of eye rubbing alone.

The immune response to alloantigen consists of an afferent and an efferent arm. In the afferent arm, APCs travel from the graft-bearing alloantigen to regional lymph nodes, where they are presented to T cells. Increased expression of MHC class II in the cornea has been described in experimental allergic conjunctivitis,²⁵ raising the possibility that alloantigen recognition in the afferent limb may be enhanced in Sens⁺Chall⁺ recipients of allografts. The efferent arm culminates in infiltration and destruction of the graft by a variety of effector cells. We have shown that perioperative allergic conjunctivitis influences the effector arm of the immune response in that it is associated with an eosinophilic infiltrate during graft rejection. Graft infiltration by eosinophils has been previously described in rejected human allografts in patients with allergic conjunctivitis.²⁶ Eosinophilic infiltration is a prominent feature of unmodified rejection in corneal and pancreatic xenotransplants.^{27–29} In animal models of skin and cardiac allotransplantation, eosinophilic infiltration is seen characteristically in Th2-biased animals.^{17,30}

Three questions must be addressed regarding eosinophils: Are they specifically recruited to the cornea during graft rejection? Do they contribute to graft destruction? Are they responsible for the increased rate of graft rejection?

One possible route of alloreactive cell trafficking in rejection to graft stroma is by way of the surrounding conjunctiva. Another is to the graft endothelium by way of the ciliary body and iris through the anterior chamber. Eosinophils that enter the cornea and anterior chamber in allergic eyes appear to do so as part of a specific response to alloantigen, an observation supported by the absence of eosinophils in isograft recipient eyes with allergic conjunctivitis despite their presence in the conjunctiva. The capacity of eosinophils in parasitic and allergic inflammation to initiate and sustain an inflammatory response is largely caused by the release of cationic proteins, including MBP (anti-MBP antibody is used to identify eosinophils in tissue sections³¹), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN or EPX). These proteins can directly injure mammalian cells and can induce cytokine and chemokine release from bystander cells. Eosinophils certainly have the capacity to injure the graft, but their importance as effector cells in corneal allograft rejection remains undetermined. That said, of the CD4 cells, CD8 cells, and macrophages that have been consistently found in the infiltrate of rejected grafts,^{32,33} only CD4 cells have been shown to play an essential role in the rejection process.⁹ Although graft-infiltrating eosinophils were seen exclusively in allergic conjunctivitis, we found their absolute number to be less than those of CD4 cells, CD8 cells, or macrophages. Further studies are required to ascertain the functional importance of eosinophils as effector cells in graft rejection and whether they contribute to the accelerated rate of rejection seen after perioperative allergic conjunctivitis.

In the model we report, the induced allergy was at its most severe very early in the postoperative course, but this inflammation had far-reaching effects on graft survival. It is possible that the allergen-induced conjunctival inflammation, immediately after transplantation, may influence the afferent and the efferent limb of the allogeneic response. In avascular recipient corneas, the indirect route of alloantigen presentation is thought to be predominant,³⁴ with APCs migrating to the graft from recipient conjunctiva and limbus. It may be that phenotypic or functional alterations in conjunctival APC in allergy alter the afferent component of the rejection response.

In summary, we have shown that allergic conjunctivitis at the time of corneal allotransplantation significantly shortens corneal allograft survival. The increased rate of graft rejection after perioperative allergic conjunctivitis is correlated with an eosinophilic infiltrate of the graft and anterior uvea at the time of rejection, but this may be only one aspect of the allogeneic response to a graft in allergic eye disease in addition to that found in its absence.

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