Effect of Immunosuppression on Outcome Measures in a Model of Rat Limbal Transplantation

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PURPOSE. To examine the effect of immunosuppression with intramuscularly injected cyclosporine and topical corticosteroid on limbal allograft survival in a new model in the inbred rat.

METHODS. Orthotopic limbal tissue harvested from male Fischer 344 (isografts) or male Wistar-Firth donors (allografts) was sutured into superior and inferior lamellar excision sites in female recipient Fischer 344 rats. Grafts were examined clinically for at least 55 days. Superficial epithelial cells were sampled weekly, and the DNA extracted and probed for the male-specific gene Sry by polymerase chain reaction. Recipients were killed at established intervals for immunohistochemistry. Graft-recipient animals were randomly assigned to receive either intramuscular cyclosporine plus topical prednisolone phosphate or vehicle for 4 weeks from the time of transplantation.

RESULTS. Isografts survived for a median of more than 55 days. Allografts underwent clinical rejection at a median of 6 to 7 days after grafting. Acutely rejecting allografts showed a dense mononuclear infiltrate consisting of activated CD4+ and CD8+ T cells with some macrophages. Genomic Sry was usually detectable in cells sampled from the ocular surface for more than 55 days in isografts, but not beyond 7 days in allografts. Immunosuppression prolonged allograft survival significantly, as assessed clinically, but did not prolong donor cell survival on the ocular surface, as assessed by detection of genomic Sry.

CONCLUSIONS. A robust model of limbal transplantation was developed in the rat. Isografts survived for the long term, whereas allografts underwent rapid rejection. Although clinical allograft survival was prolonged to a modest extent by immunosuppression, donor cell survival on the ocular surface was not improved. (Invest Ophtalmol Vis Sci. 2002;43:647–655)

Limbal transplantation is used increasingly to treat ocular surface disease, but the biological behavior of these grafts is poorly understood. The limbal epithelium is believed to contain the stem cells of the corneal epithelium.1,2 When the limbus is damaged or becomes dysfunctional, a recognizable pattern of corneal surface disease occurs that has been termed limbal failure or limbal dysfunction.3,4 The surface of the cornea in patients with limbal dysfunction becomes vascularized, inflamed, and opaque and is prone to recurrent painful corneal erosions. These changes are irreversible and damage vision.

Transplantation of the limbus to treat limbal failure is based on the premise that the transplanted donor stem cells will repopulate the corneal epithelium and remain functional in the long term.5,6 Unilateral disease can be treated by an autograft of healthy limbus taken from the other eye.7–10 There is a risk, however, that limbal dysfunction may develop at the donor site of a clinically normal eye or that limbal autografts may function poorly because of subclinical disease.10,11 Furthermore, patients who have bilateral limbal dysfunction cannot be treated with autografts and require limbal allografts from a donor eye.10,12–18

Theoretically, limbal allografts should undergo immunologic rejection, because the limbus and peripheral cornea contain many antigen-presenting dendritic cells.19 Rejection reactions in limbal allografts have been described clinically,5,10,14,15,16,20–23 but the appearance of these rejection episodes varies widely and may be difficult to diagnose with certainty. Rejection of limbal grafts probably destroys donor stem cells so that the corneal epithelium can no longer be repopulated by cells of donor origin. Donor cells are difficult to demonstrate on the recipient ocular surface, even after apparently successful limbal allografts.24–26 This may be due to a lack of test sensitivity or may alternatively be a true reflection of graft failure. In addition, the differences in protocols for postoperative care and immunosuppression make the detection and interpretation of immune rejection difficult. There are no clear guidelines as to the most effective strength, frequency, duration, or efficacy of postoperative immunosuppression.

We report a model of limbal transplantation in the inbred rat that is robust and technically similar to the procedure performed in clinical practice. We used this model to observe the behavior of untreated grafts and, more important, to investigate the influence of a regimen of immunosuppression on graft and donor cell survival.

METHODS

Experimental Animals

Inbred adult Fischer 344 (F344) and Wistar-Firth (WF) rats were bred within our institution and allowed unlimited access to water and rat chow. All operative procedures were performed with rats under general anesthesia. Use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgical Technique of Limbal Transplantation in the Rat

Donor rats were killed with an overdose of inhalational halothane. Circumferential lamellar grafts containing limbal epithelium were excised from the superior and inferior limbus of each donor eye and stored in sterile balanced salt solution (BSS; Alcon, Frenchs Forest, New South Wales, Australia) at room temperature. Grafts had a chord length of approximately 5 mm and were approximately 2 mm wide—thus containing some peripheral cornea and a conjunctival frill. Recipient rats were anesthetized with halothane, and graft sites were marked at the superior and inferior limbus of the right eye, using the donor grafts as templates. A diamond knife was used to make a lamellar limbal excision of slightly smaller size at each site to create an inlay bed for
the graft (Fig. 1). Grafts were sutured into the recipient beds with eight interrupted 10-0 nylon sutures (Alcon). The knots remained exposed. The recipient cornea was kept moist by frequent irrigation with BSS. Chloramphenicol ointment 1% (Parke-Davis Pty. Ltd., Caringbah, Australia) was placed into the conjunctival sac of the grafted eye at the end of surgery.

Postoperative Management and Clinical Graft Assessment

Surgically treated eyes received 1 drop of 0.5% chloramphenicol (Parke-Davis Pty. Ltd.) daily for 3 days. Sutures were not removed. Graft-recipient eyes that showed signs of infection were excluded from the study ($n = 2$). Recipients were examined each day at the slit lamp microscope for a maximum of 65 days after grafting. A single masked observer numerically scored each graft at the slit lamp microscope for a maximum of 63 days after grafting. A single masked observer numerically scored each graft at the slit lamp microscope according to the following criteria. Graft thickness and graft inflammation were assessed separately on a scale of 0 to 3 in 0.5-point increments. Graft thickness was scored as 0 (normal thickness), 1 (mild edema), 2 (moderate edema), and 3 (severe edema); graft inflammation was scored as 0 (not inflamed), 1 (mild inflammation), 2 (moderate inflammation), and 3 (severe inflammation). The degree of corneal vascularization was also recorded. Each graft was allocated a rejection score derived by summing the scores for graft thickness and graft inflammation. Clinical graft rejection was defined as a rejection score of 3.5 or more after at least 2 days of rejection scores of 2.5 or less. Scores on the first day after surgery were excluded.

Detection of Donor Cells on the Ocular Surface

Superficial epithelial cells from the ocular surface of grafted eyes were sampled at various time points after surgery. Recipients were anesthetized, and samples were taken from central cornea and from directly over each graft by gentle debridement, using 3 mm discs of DNA-processing paper (FTA; Fitzco Inc, Maple Plain, MN). Two discs containing recipient epithelial samples were collected from each site; a total of six samples per eye. In selected donor rats, samples were collected from the limbal sites being used as grafts. The discs were placed directly into polymerase chain reaction (PCR) tubes (Perkin Elmer, Branchburg, NJ) and processed according to the manufacturer's instructions. Briefly, each disc was washed three times in purification reagent (FTA Purification Reagent; Fitzco Inc) and then washed twice in TE buffer (10 mM Tris-Cl [pH 8.0]; 0.1 mM EDTA [pH 8.0]) before being air dried at 60°C for 1 hour. To detect the presence of male donor cells on the ocular surface of female recipients, the first purification-bath-treated disc from each collection site was processed by PCR for the amplification of Sry.

As a positive control for presence of DNA, the second disc from each collection site was processed for amplification of the oncogene N-ras. The primer sequences for Sry were 5'-CCC GCG GAG AGA GGC ACA AGT-3' and 5'-TAG GGT CTT CAG TCT CTG CGC-3', giving a product size of 146 bp. The primer sequences for N-ras (the gift of Sim Neoh, Flinders Medical Center, Adelaide, Australia) were: 5'-TGA AGA AGC ACA AGA CTA GCC-3' and 5'-TGA TAT GGT GGT ATC ATA TTC A-3', yielding a product size of 110 bp. Each PCR reaction contained 20 ng of each primer, 10 µL of 5× PCR buffer (500 µM dNTPs, 10 mM MgCl2, 50 mM Tris-Cl [pH 8.3], and 250 mM KCl), 0.5 U of DNA polymerase (TaqGold; Perkin Elmer), and one purification-bath-treated disc in a final reaction volume of 50 µL. Amplification conditions were as follows: 1 cycle denaturation (95°C, 5 minutes), 60 cycles annealing (55°C, 10 seconds), extension (72°C, 5 minutes), and termination (72°C, 5 minutes), and extension (72°C, 5 minutes). PCR amplification products were resolved by electrophoresis in 3% agarose/Tris-borate gels containing 0.5 µg/mL ethidium bromide and visualized under ultraviolet illumination.

Immunohistochemistry

Graft-recipient eyes were enucleated and immersion fixed in paraformaldehyde-lysine-periodate, snap frozen in liquid nitrogen, and cryostat sectioned at 8 µm. Immunoperoxidase staining was performed as described elsewhere. Primary monoclonal antibodies were produced as undiluted supernatants from stationary phase hybridomas. Hybridomas X63 (unknown specificity), the IgG1 isotype negative control; OX-1 (anti-CD4), OX-35 (anti-CD4), OX-8 (anti-CD8), NDS-61 (anti-CD25), and R73 (anti-Tαβ cell receptor), OX-6 (anti-major histocompatibility complex [MHC] class II), and OX-18 (anti-MHC class I), and
Autografts, female 344→self
Isografts, female F344→female F344
Isografts, male F344→female F344
Allografts, female WF→female F344
Allografts, male WF→female F344

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<th>Graft Strain and Gender Disparity</th>
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<th>n</th>
<th>Day of Clinical Rejection</th>
<th>Median Day of Rejection</th>
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<td>7†</td>
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* No significant difference in the day of rejection when male donors were compared with female donors (P > 0.05).
† The median day of rejection for allografts was significantly different from that of isografts (P = 0.0001, Mann-Whitney test corrected for ties).

Experimental Groups
All experimental groups contained at least six recipients. Limbal autografts were performed in F344 female rats. Orthotopic isografts were transplanted from F344 donors to female F344 recipients. Orthotopic allografts were transplanted from WF donors to female F344 recipients across multiple minor and major histocompatibility antigen disparities. Gender mismatching of donors and recipients allowed male donor cells to be tracked in female recipients after transplantation. Same-sex allografts and isografts were also performed to determine whether gender-related minor histocompatibility differences influences graft outcome. To examine the influence of immunosuppression, sex-mismatched limbal allografts and isografts were performed as described. Graft-recipient animals were randomly assigned to treatment with intramuscular injection of cyclosporine (20 mg/kg three times weekly) plus topical prednisolone phosphate 0.5% (Minims; Smith & Nephew Pharmaceuticals Ltd., Romford, UK), one drop applied to the grafted eye three times per week. Control groups received drug vehicle three times per week according to the same protocol. Cyclosporine powder (Novartis; Basel, Switzerland) was dissolved by vigorous shaking at 70°C in sterile-filtered peanut oil (Orion, Perch, Western Australia) to a final concentration of 50 mg/mL. Treatment began on the day before surgery and continued for 4 weeks after surgery.

Statistical Analyses
Statistical analyses were performed on computer (SPSS, ver. 6.1; SPSS Science, Chicago, IL). Comparisons of nonparametric data were made using the Mann-Whitey test corrected for ties and the two-tailed Fisher exact test. P < 0.05 was considered significant.

Results
Outcome of Limbal Transplantation in Nonimmunosuppressed Recipients
Autografts and isografts both survived indefinitely (>55 days, Table 1). There was no significant difference between isografts and autografts with respect to the degree of postoperative graft thickness or inflammation (P > 0.05). In contrast, most allo-

Rat Limbal Allograft Rejection 649

Effect of Immunosuppression on Limbal Graft Outcome
Cohorts of male-to-female isografts and allografts were then treated with intramuscular cyclosporine and topical prednisolone phosphate, or with drug vehicle according to the same protocol (Table 2). All limbal isografts survived for the
long term (>63 days), and neither the vehicle nor the immunosuppressive treatment affected outcome in any discernible way. Both superior and inferior limbal allografts in recipients treated with vehicle underwent clinical rejection at a median of 6 days after transplantation, in a time frame that did not differ significantly from that observed in untreated recipients.

Figure 2. Detection of male donor cells in epithelial samples taken from female recipients with limbal isografts (A) or allografts (B) at various postoperative time points. PCR was used to amplify genomic Sry in DNA extracted from each sample. Samples were taken from over the superior graft (□, ◦), the inferior graft (□, ■), and the central cornea (□, △). Open symbols: samples from isografts or allografts that did not undergo clinical rejection; filled symbols: allografts that underwent clinical rejection. Each symbol represents the result in one of three samples from one grafted eye.

Figure 3. Immunohistochemistry on representative limbal grafts. (A) Allograft stained for CD4 during acute clinical rejection, showing a dense mononuclear cell infiltrate and many CD4-positive cells. (B) Time-matched isograft stained for CD4. Inflammatory infiltrate was minimal. Magnification, ×320.
Table 2. Clinical Assessment of Limbal Graft Survival in Immunosuppressed Recipients

<table>
<thead>
<tr>
<th>Graft Strain, Gender Disparity, and Treatment Group</th>
<th>Graft Position</th>
<th>n</th>
<th>Day of Clinical Rejection</th>
<th>Median Day of Rejection</th>
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<td>&gt;63</td>
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<td>Isografts + immunosuppression, male F344→female F344</td>
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<td>&gt;63, &gt;63, &gt;63, &gt;63, &gt;63, &gt;63</td>
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<td></td>
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<td>&gt;63, &gt;63, &gt;63, &gt;63, &gt;63, &gt;63</td>
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<td>Allografts + vehicle, male WF→female F344</td>
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<td>7</td>
<td>5, 6, 6, 6, 7, 7, &gt;63</td>
<td>6</td>
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<tr>
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<td>8</td>
<td>6, 6, 6, 6, 6, 7, 7</td>
<td>6</td>
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<tr>
<td>Allografts + immunosuppression, male WF→female F344</td>
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<td>5, 6, 6, 7, 8, 14, &gt;63, &gt;63</td>
<td>7.5</td>
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<td>35.5</td>
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+ immunosuppression = 20 mg/kg intramuscular cyclosporin A plus topical 0.5% prednisolone phosphate, delivered three times weekly for 4 weeks from the day before graft; + vehicle = similar treatment, but with drug vehicle.

\(P > 0.05\). Allograft recipients treated with intramuscular cyclosporine and topical prednisolone phosphate underwent clinical rejection at a median of 7.5 days after transplantation for superior grafts and 35.5 days after for inferior grafts—that is, inferior grafts appeared to show a slightly better clinical outcome than did superior grafts. There was a significant difference in inferior graft survival, as assessed clinically, between immunosuppressed and vehicle-treated recipients \((P = 0.02)\), but there was no significant difference in superior graft survival between immunosuppressed and vehicle-treated recipients \((P = 0.41)\). Overall, immunosuppression increased the proportion of long-surviving allografts compared with vehicle-treated control grafts, at least as assessed clinically \((P = 0.02)\); nonetheless, the majority of allografts underwent rejection.

In epithelial samples taken from donor and recipient eyes for the detection of N-ras, 750 (98.5%) of 741 were positive. In isografts, Sry was usually detectable over the grafts and central cornea for the entire observation period in both vehicle-treated (88% samples positive for Sry) and immunosuppressed recipients (93% samples positive for Sry; Figs. 4A, 4B). In vehicle-treated allografts (control group), Sry was generally not detected on the ocular surface after onset of clinical rejection, with only 10 (5%) of 200 samples returning a positive result (Fig. 4C). In immunosuppressed allografts (test group), the rate of detection of Sry decreased rapidly in the second week after surgery (Fig. 4D), with only 17 (8%) of 208 samples showing evidence of the presence of Sry after postsurgical day 7. In a few instances, donor cells were still detected on the ocular surface in eyes assessed as having undergone clinical rejection of grafts. In most cases, however, donor cells were detectable before the time of rejection in vehicle-treated rats and were undetectable thereafter, regardless of whether such grafts had undergone clinically apparent rejection.

**Discussion**

We set out to develop a clinically relevant model of limbal transplantation in the inbred rat in which clinical outcome could be correlated with the detection of donor cells on the ocular surface and in which the influence of immunosuppression on graft survival could be investigated. The model, in which superior and inferior lamellar limbal grafts were inlaid into prepared recipient beds, was designed to mimic a commonly used clinical procedure of limbal transplantation as closely as possible. It was not designed to treat limbus-deficient eyes, in the first instance, which is a limitation of the study. Attempts to create a limbus-deficient rat recipient resulted in an ethically unacceptable rate of suppurative keratitis.

Eighty percent of limbal allografts in otherwise untreated rat recipients showed a clinical rejection response characterized by increasing graft edema and inflammation at 1 to 2 weeks after surgery. The appearance of clinical rejection coincided with a dense inflammatory cell infiltrate in grafted tissue, as assessed by immunohistochemistry. The infiltrate exhibited typical characteristics of a delayed-type hypersensitivity response, including presence of activated T cells, macrophages, and granulocytes and upregulation of MHC antigens. In contrast, neither autografts nor isografts exhibited a clinical rejection response, and histologic analysis showed no significant inflammatory infiltrate. Genomic Sry, a marker of the presence of male donor cells,\(^27\) was readily detected on the ocular surface of most female recipients of isografts for as long as the grafts were observed, but could seldom be detected on the ocular surface of allografts after the time of clinically apparent rejection, even though some allografts did not appear to undergo clinical rejection. This suggests that subclinical allograft rejection may have occurred in some instances. However, within the limits of the detection system, the presence or absence of donor cells on the ocular surface after transplantation correlated reasonably well with clinical outcome in unmodified recipients.

An immunosuppressive regimen combining relatively high-dose intramuscular cyclosporine with topical corticosteroid produced a significant, albeit biologically marginal, prolongation of limbal allograft survival. Intramuscular cyclosporine and topical prednisolone were chosen because they are frequently used in clinical practice\(^10,13,15–18,24,30–33\) and in experimental studies\(^34,35\) of limbal allotransplantation. A dose of 20 mg/kg cyclosporine administered intramuscularly three times weekly for 4 weeks was selected on the basis that lower doses have been shown to be highly effective in modulating rejection of a variety of allografts in the rat\(^36–38\) and that a dose of 20 mg/kg · d is approximately the maximum tolerated dose in this species.\(^39\) Topical prednisolone was given three times a week, because more frequent doses in rats can cause anorexia and weight loss.\(^40\) The better clinical survival of inferiorly placed allografts compared with superiorly placed grafts was surprising. We speculate that pooling of topical corticosteroid in the inferior tear film or conjunctival fornix, which increases drug contact time with the inferior graft, may have served to dampen the inflammatory response. However, the majority of allografts underwent clinical rejection, despite immunosuppression. Furthermore, donor genomic Sry was undetectable on the ocular surface of most allografted eyes from the second postoperative week and, as in unmodified recipients, donor cells could not be detected in some immunosuppressed rats, despite the absence of significant clinical signs of rejection. Our data indicate that the substantial amount of immunosuppression administered may have exerted a modest beneficial effect on the clinical appearance of the limbal allografts, but that this effect was not translated into improved donor cell survival on the ocular surface. We suggest that the monitoring of donor cell survival on the ocular surface represents a better method of assessing graft survival than does clinical appear-
ance, especially in recipients in which immunosuppression has been administered.

Many other investigators have examined experimental limbal allograft outcome in animal models, but there are relatively few reports involving the use of inbred rodents, in which histocompatibility differences between donor and recipient can be readily controlled. Sone used a rat model of keratoplasty to show that the regenerating epithelium after allograft rejection appeared to be of recipient conjunctival origin. Yao et al. developed a model in which a full-thickness corneal lenticule was transplanted to the limbal region of the mouse eye, and used it to demonstrate that recipient corneas could be re-epithelialized by graft-derived epithelium and that untreated grafts underwent rejection. The effect of immunosuppression on graft survival was not tested in these studies. The model described herein differs from the murine model, in that partial-thickness limbal tissue (without donor endothelium) was inlaid into prepared beds, such that donor and recipient epithelia were contiguous.

The long-term success of human limbal allografts is poor by most transplantation standards with clinical success rates of only 50% at 3 years. Rejection and loss of donor corneal epithelial stem cells probably account in part for this poor result, but outcome after clinical limbal transplantation can be difficult to assess. Using fluorescent in situ hybridization and restriction fragment length polymorphism analyses, Shimazaki et al. demonstrated long-term survival of donor cells on the corneal surface in 7 of 10 eyes after human limbal allograft transplantation, with follow-up ranging from 12 to 30 months after transplantation. All but one of these eyes also had had a penetrating corneal graft from the same donor, and all patients had received topical and systemic immunosuppression. Clinically, however, there was no difference in outcome among eyes with or without donor-derived epithelium. In a single

FIGURE 4. Effect of immunosuppression on detection of male donor cells in epithelial samples from female recipients with limbal isografts or allografts. PCR was used to amplify genomic Sry in DNA extracted from each sample. Symbol descriptions are as in Figure 2. (A) Isografts treated with drug vehicle; (B) isografts treated with immunosuppression; (C) allografts treated with drug vehicle; (D) allografts treated with immunosuppression.
patient treated with systemic and topical immunosuppression and observed for 5 months, we were unable to detect donor cells on the ocular surface beyond 20 weeks after transplantation by DNA microsatellite analysis, despite an apparently successful graft, as judged by the clinical appearance. More recently, we reported modest objective clinical benefit in five patients observed for 3 to 5 years after limbal allotransplantation, but in whom no donor cells were detectable on the ocular surface by DNA fingerprinting analysis.26

The discrepancy between apparent clinical improvement and absence of donor-derived corneal epithelium after limbal allotransplantation has been difficult to interpret. Experience with limbal autografts,10,11 grafts from living-related donors,23 and retrospectively HLA-typed grafts43 suggest a clear relationship between avoidance of inflammation or immunologic rejection and good postoperative function. A wealth of evidence suggests that limbal allograft survival is compromised by the occurrence of a rejection reaction.5,10,14,15,18,20–24,41,42 Limbal allograft rejection presumably destroys corneal epithelial stem cells or their more differentiated progeny. Our experimental data plainly show the connection between donor cell survival and absence of rejection. Donor cells were frequently detected on the ocular surface of isografts in the longer term. This also suggests that the remaining recipient limbal cells, located nasally and temporally, do not overwhelm surviving donor cells. Donor cells were difficult to detect in allografts after the onset of rejection, and immunosuppression reduced donor cell attrition to some extent. Donor cell survival on the ocular surface may not be essential for some clinical improvement after limbal transplantation, but the presence of such cells is a useful indicator that rejection has been largely circumvented. Clearly, however, rejection is not the only reason for limbal graft failure. Regional factors such as epithelium-fibroblast interactions,44 corneal basement membrane,45 the degree of neural innervation,46 and stromal inflammation47 may all affect corneal epithelial cell proliferation and differentiation. Adequate tear function is necessary for successful limbal allograft transplantation in patients with severe Stevens-Johnson syndrome.48

Is immunosuppression obligatory for limbal allograft survival, and, if so, what regimen should be used? We argue that rejection reactions are common and may go unrecognized at
clinical examination. Should the function of limbal grafts be dependent on survival and differentiation of donor corneal epithelial stem cells, then intervention would be needed to prevent rejection. The most appropriate regimen of immunosuppression in human patients remains uncertain. Existing protocols may be insufficient to sustain donor cell survival. The experimental model we describe may prove useful in testing suitable alternatives.

**Acknowledgments**

The authors thank Pan Sykes for helpful discussions, Scott Standfield for technical assistance, and Ray Yates for animal husbandry.

**References**


