Anterior Chamber-Associated Immune Deviation Promotes Corneal Allograft Survival

Jerry Y. Niederkorn and Jessamee Mellon

Purpose. To determine whether anterior chamber-associated immune deviation (ACAID) promotes corneal allograft survival.

Methods. CB6F1 mice were grafted with orthotopic corneal transplants from C3H donors (mismatch at the entire major histocompatibility complex plus multiple minor histocompatibility loci) and from NZB donors (mismatch only at multiple minor histocompatibility loci). ACAID was induced by priming in the anterior chamber (AC) with either Ia− spleen cells, Ia+ spleen cells, corneal endothelial cells, or corneal epithelial cells from corneal allograft donors before orthotopic transplantation. The role of ACAID in promoting corneal allograft survival was examined by determining the fate of corneal allografts in splenectomized and eusplenic mice.

Results. Anterior chamber priming produced a modest enhancement of the survival of fully allogeneic C3H corneal allografts. By contrast, AC priming with Ia− NZB spleen cells or NZB corneal endothelial cells results in the permanent acceptance of NZB corneal grafts in 60% and 90% of the CB6F1 hosts, respectively. Abolition of ACAID by splenectomy resulted in a sharp increase in the incidence of graft rejection in donor-host combinations involving multiple minor histocompatibility disparity.


Corneal allografts are the oldest, most common, and arguably the most successful solid tissue grafts. In the United States, more than 40,000 corneal transplantations are performed each year, and, of this number, fewer than 10% will fail.1 The high success rate of keratoplasty suggests that corneal grafts enjoy a degree of immunologic privilege not shared by other categories of grafts.2,3 A dramatic example of this privilege is shown when one compares the fate of skin allografts with orthotopic corneal allografts transplanted across major histocompatibility complex (MHC) and multiple minor histocompatibility barriers in rodents. Such "fully allogeneic" skin allografts typically are rejected in 100% of the hosts.4,5 By contrast, corneal allografts transplanted across similar barriers are rejected in only 50% to 55% of the hosts.6–8

The immunologic privilege of corneal allografts is referable to several unique features of the cornea and eye. The avascular nature of the corneal graft bed sequesters corneal alloantigens and thereby establishes afferent blockade of the immune response. The paucity of MHC class I and II antigens on the corneal endothelium renders the corneal allograft antigenically invisible and contributes to afferent blockade of the immune response.2,3 Moreover, the absence of resident antigen-presenting cells, namely Langerhans cells, in the central corneal epithelium diminishes the immunogenicity of corneal allografts and reduces their risk for immunologic rejection.2,3,9

Corneal grafts also may benefit from their proximity to the anterior chamber of the eye, well recognized as an immunologically privileged site. In fact, the endothelium of the corneal allograft forms the anterior
lining of the anterior chamber of the eye. Immunologic privilege of the anterior chamber of the eye is caused, at least in part, by an aberrant systemic immune response termed anterior chamber-associated immune deviation (ACAID) induced when antigens such as alloantigens are introduced into this ocular compartment. 2,10 Intracameral inoculation of alloantigens results in the active suppression of delayed-type hypersensitivity (DTH) but the preservation of normal cytotoxic T lymphocyte (CTL) and humoral antibody responses. 2,10 Moreover, anterior chamber presentation of alloantigens promotes the long-term survival of orthotopic skin allografts. This latter finding led Sonada and Streilein 11 to suspect that corneal allografts might induce ACAID and thereby enhance their own survival. These investigators reported that mice bearing long-term corneal allografts displayed a suppression of alloantigen-specific DTH, whereas mice that had rejected their corneal allografts coincidently developed DTH to donor alloantigens. These results suggest that the fate of orthotopic corneal allografts is affected significantly by their capacity to induce ACAID. She and coworkers 12,13 provided even more compelling evidence that induction of ACAID could have beneficial effects on corneal allograft survival. Their results indicated that anterior chamber inoculation of allogeneic cells could reduce rejection from 80% in untreated rats to as low as 15% in rats primed in the anterior chamber with donor-derived, B-cell-enriched cell suspensions. 18 In a subsequent study, Yao et al 14 demonstrated that anterior chamber priming with donor-specific lymphocytes reduced the incidence of orthotopic corneal allograft rejection in a rat model of penetrating keratoplasty. In the current study, we wanted to determine whether induction of ACAID by intracameral injection of allogeneic corneal cells or allogeneic lymphoid cells would enhance the survival of subsequent corneal allografts, especially in high-risk, previously sensitized hosts. Similarly, we wondered whether splenectomy, an effective method for abolishing the induction of ACAID, would jeopardize the survival of corneal allografts in otherwise normal hosts.

MATERIALS AND METHODS

Animals

Female NZB (H-2d), C3H (H-2k), and CB6F1 (H-2b/ d) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and were used between the ages of 2 and 8 months. The use and treatment of mice in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Alloantigen Preparation

Murine corneal epithelial and endothelial cells were isolated from freshly dissected corneal explants 1,5,16 and propagated in minimal essential medium supplemented with 10% fetal calf serum. After the primary cultures were established, these cells were immortalized with human papilloma virus genes E6 and E7 using the disabled recombinant retroviral vector PLXSN16E6/E7. These cells proliferate indefinitely while they maintain their original morphologic characteristics. 17 Furthermore, the cells express the same histocompatibility antigens as their nontransformed counterparts (unpublished data, 1996).

Previous studies 18 have shown that Ia+ lymphoid cells were superior to Ia+ cells in the induction of ACAID against minor histocompatibility antigens. Therefore, Ia+ and Ia+ lymphoid cells were compared for their capacity to promote corneal allograft survival. Spleen cell suspensions enriched for either Ia+ or Ia+ cells were prepared as previously described. 18 Briefly, spleen cells were pressed through a fine stainless steel sieve. Erythrocytes were removed with a 4-minute incubation in ammonium chloride–KH2PO4 solution at 37°C, followed by washing three times in Hank’s balanced salt solution (HBSS). Adherent cells were separated by incubating cell suspensions (5 X 106 cells/ml) on plastic tissue culture dishes (Falcon 3803; Becton Dickinson, Oxnard, CA) at 37°C in 5% CO2 for 90 minutes. Nonadherent cells were washed gently with HBSS, and adherent cells were collected by gentle scraping with a Teflon policeman (#3010; Costar, Cambridge, MA) and rinsing with HBSS. Adherent spleen cell suspensions prepared in this manner contained >70% Ia+ cells and nonadherent cell suspensions contained approximately 15% Ia+ cells.

Langerhans Cell Induction

The central corneal epithelium of the mouse is normally devoid of resident Ia+ Langerhans cells (LC), which usually are situated in the epithelium of the limbus. 19 However, LC can be induced to migrate centripetally from the limbus to the central corneal epithelium by the instillation of sterile latex beads (1 μm diameter; Sigma Chemical, St. Louis, MO) into shallow incisions in the corneal epithelium. 19 Corneal allografts (2.5 mm diameter) were prepared from NZB corneas treated with sterile latex beads 7 days earlier. Langerhans cell migration into the central cornea was confirmed by immunofluorescence and adenosine triphosphatase staining as described elsewhere. 19

Orthotopic Corneal Transplants

Full-thickness penetrating NZB (H-2b) or C3H (H-2k) corneal grafts (2.5 mm diameter) were transplanted
orthotopically onto the right eyes of anesthetized CB6F1 (H-2b/d) mice using a procedure previously described by She et al.\(^20\) and modified by He et al.\(^21\) Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (1 to 2 mg/mouse; Abbott Laboratories, Chicago, IL). Topical anesthetic (Alcon Laboratories, Fort Worth, TX). Both the donor graft and the recipient graft bed were scored with 2.5-mm and 2.0-mm diameter trephines, respectively (Storz Instruments, St. Louis, MO) before removal of the corneal button using vannas scissors (Storz). The donor graft was sewn into place using 12 interrupted 11-0 nylon sutures and a 50-µm diameter needle (2881G; Ethicon, Somerville, NJ). Sutures were removed 7 to 8 days later. Topical antibiotic (Ocumycin; Bausch & Lomb, Tampa, FL) was applied after surgery. No immunosuppressive drugs were used.

**Clinical Observations**

Grafted eyes were examined with a slit lamp biomicroscope at least twice a week throughout the entire study period. Graft opacity, edema, and neovascularization were scored as minimal, moderate, or severe, as previously described.\(^22\) If all three parameters became moderate or severe more than 7 days after transplantation, the graft was recorded as rejected on that day. Any host that developed complications such as cataact, anterior chamber loss, iris synechiae, or infection, was excluded from the study. Results are expressed as mean survival time ± standard deviation of the mean. The Mann–Whitney test was used to determine the statistical significance of the results.

**Heterotopic Corneal Transplants**

Heterotopic transplantation of LC-containing grafts is an effective method for inducing allospecific CTL and DTH responses.\(^19\) Moreover, heterotopically grafted hosts reject subsequent orthotopic corneal grafts in an accelerated fashion.\(^23\) Two LC-containing NZB limbus grafts (3.0-mm diameter) were implanted into subcutaneous pockets in CB6F1 mice. The incisions were closed with stainless steel staples. Staples were removed 3 weeks later, and the mice were challenged with orthotopic NZB grafts.

**Anterior Chamber Priming**

The technique for transplanting alloantigenic cells into the anterior chamber of the mouse eye has been described elsewhere.\(^18\) A Hamilton (Whittier, CA) automatic dispensing apparatus was used to dispense 5 µl of cell suspensions into the AC of anesthetized mice. Anterior chamber inocula consisting of either 1 X 10^5 nonadherent or adherent NZB or C3H spleen cells were injected into the left eyes of CB6F1 mice. In other experiments, 1 X 10^5 NZB corneal epithelial or endothelial cells were injected into the AC of the left eyes of CB6F1 mice.

**Delayed-Type Hypersensitivity Assay**

Delayed-type hypersensitivity responses to alloantigens were measured by a conventional footpad swelling assay.\(^19\) CB6F1 mice were primed in the AC on day −7 with either 1 X 10^5 NZB corneal endothelial cells, 1 X 10^5 NZB corneal epithelial cells, or 1 X 10^5 nonadherent NZB spleen cells. On day 0, all the AC primed mice were immunized with a subcutaneous injection of 2 X 10^5 NZB spleen cells. Two weeks later, all mice—including untreated, negative control mice—were assessed for footpad swelling responses to NZB alloantigens. Both hind footpads of each mouse were measured with an engineer’s micrometer (Mitutoyo, Tokyo, Japan) immediately before footpad challenge. An eliciting dose of 1 X 10^7 gamma-irradiated (3000 cGy) NZB splenocytes suspended in 25 µl of HBSS were injected into the subcutaneous tissue of the right hind footpad. The left hind footpad served as a background control and was administered 25 µl of HBSS without splenocytes. Both footpads were measured 24 hours later, and the difference in footpad swelling size was used as a measure of DTH. Results were expressed as specific footpad swelling, which equals [(24-hour right hind foot measurement − 0-hour right hind foot measurement) − (24-hour left hind foot measurement − 0-hour left hind foot measurement)] X 10^-4 ± SEM in inches. Student’s t-test was used to evaluate the statistical significance of the results.

**Corneal Allograft Models**

Previous investigations have demonstrated that ACAID is induced more readily with minor histocompatibility (H) antigens compared to MHC antigens.\(^24\) Moreover, the induction of ACAID in donor–host combinations representing combined histoincompatibility at multiple minor H and MHC loci is transient, whereas ACAID induced with multiple minor H antigens alone is long-lived.\(^25\) Therefore, two donor–host combinations were used. C3H donor grafts transplanted to CB6F1 recipients represents histoincompatibility at the entire MHC, as well as multiple minor H loci; for convenience, they are designated as fully allogeneic. NZB donor grafts share the same MHC genotype as the CB6F1 recipients but differ with the host at all minor H loci. Thus, the NZB corneal grafts are designated minor H disparate corneal allografts.

Studies have demonstrated that in both rats and mice, only 26% to 53% of minor H disparate orthotopic corneal allografts undergo immunologic rejection\(^26\) unless pretreated with stimuli that induce the centripetal migration of donor-derived LC into donor corneas before their removal for orthotopic transplantation.\(^26\) Therefore, in most of the experi-
ACAID and Corneal Allograft Survival

Anterior Chamber Priming With Alloantigenic Cells Prolongs Corneal Allograft Survival

Anterior chamber priming with either Ia~ or Ia+ C3H lymphoid cells resulted in a significant prolongation of the survival of fully allogeneic corneal allografts (Fig. 1). However, all the C3H corneal allografts eventually underwent rejection. By contrast, AC priming was found to enhance significantly the survival of minor H disparate corneal allografts. Anterior chamber priming with Ia~ NZB lymphoid cells produced a significant prolongation of LC+ NZB corneal allograft survival, although 70% of the grafts went on to reject (Fig. 2). By contrast, AC priming with Ia~ NZB cells not only prolonged graft survival but reduced the rejection rate from 80% in untreated hosts to 35% in AC-primed hosts.

Differential Effects of Corneal Epithelial and Endothelial Cells on Corneal Allograft Survival

Earlier reports suggested that corneal allograft survival correlated with the graft’s capacity to induce an antigen-specific suppression of DTH that resembled the ACAID phenotype.11 With this in mind, we assessed the effect of AC priming with allogeneic corneal cells on subsequent corneal allograft survival. CB6F1 hosts were primed in the AC with either NZB corneal endothelial cells or NZB corneal epithelial cells before receiving LC+ NZB orthotopic corneal allografts. Anterior chamber priming with NZB corneal endothelial cells produced a sharp reduction in the rejection rate and a significant prolongation in the survival time of NZB corneal grafts (Fig. 3). In untreated hosts, 87% of the NZB corneal grafts underwent rejection, with a mean survival time of 23.4 ± 16.3 days, whereas only 9% of the hosts primed in the AC with corneal endothelial cells underwent rejection. Surprisingly, AC priming with epithelial cells did not prolong significantly the survival of corneal allografts.

The failure of corneal epithelial cells to enhance the survival of either minor H disparate corneal allografts (Fig. 3) or fully allogeneic corneal allografts (data not shown) might have resulted from the inability of corneal epithelial cells to induce ACAID. This hypothesis was tested by priming CB6F1 mice with AC injections of either NZB corneal endothelial or epithelial cells on day -7. On day 0, the AC-primed mice, as well as normal CB6F1 mice, were immunized subcutaneously with NZB spleen cells. Assessment of DTH on day +14 revealed that AC priming with endothelial cells resulted in a significant inhibition in DTH responses to NZB alloantigens (Fig. 4). By contrast, CB6F1 hosts primed in the AC with NZB corneal epithelial cells failed to develop ACAID and displayed DTH responses that were of the same magnitude as hosts primed subcutaneously with NZB spleen cells.
Effect of Splenectomy on Corneal Allograft Survival

Studies in rats and mice have demonstrated that a functional, intact spleen is necessary for the development of ACAID. If the survival of corneal allografts is enhanced by the graft's capacity to induce ACAID, one would predict that disruption of the camerosplenic axis by splenectomy would jeopardize corneal allograft survival. This hypothesis was tested by assessing the survival of fully allogeneic and multiple minor H disparate corneal allografts in splenectomized CB6F1 hosts. The results showed that splenectomy had a modest, albeit insignificant, effect in hastening the rejection of C3H corneal grafts (Table 1). A more pronounced effect was seen in splenectomized CB6F1 mice grafted with LC- NZB corneal grafts: Splenectomy resulted in a significant increase in the speed of rejection of LC- minor H disparate grafts (Table 1). Moreover, splenectomy had a profound effect on the survival of LC- NZB corneal grafts. As in previous experiments, 29% of the LC- NZB grafts were rejected in eusplenic CB6F1 hosts. However, rejection rose to 91% in splenectomized hosts and occurred at an accelerated rate (mean survival time = 31.7 ± 19.0 days in splenectomized hosts versus 55.3 ± 8.1 days in eusplenic hosts).

Anterior Chamber Priming With Alloantigenic Cells Prolongs Corneal Allograft Survival in Preimmune Hosts at High Risk

Although corneal allografts enjoy a high success rate in uncomplicated first-time settings, the risk of rejection soars in hosts who previously rejected their corneal grafts. Therefore, the beneficial effects of AC priming with alloantigenic cells may reduce the risk of rejection in sensitized hosts. Accordingly, CB6F1 hosts were immunized by applying NZB heterotropic corneal allografts into subcutaneous pockets. Three weeks later, one group of mice was primed in the AC with Ia- NZB spleen cells. The other group of immunized mice was not treated through the AC. Both groups of preimmunized mice were challenged with orthotopic LC- NZB corneal allografts 1 week after the AC injection. As expected, preimmunization with heterotropic corneal allografts resulted in the accelerated rejection of all the subsequent orthotopic corneal allografts (Fig. 5). Anterior chamber priming with a single inoculum of Ia- spleen cells did not significantly enhance corneal graft survival. Therefore, a more aggressive AC priming protocol was implemented in which an additional group of CB6F1 mice was primed in the AC with three separate injections of Ia- NZB spleen cells spaced 7 days apart. As in the previous experiment, the AC-primed mice were challenged with orthotopic LC- NZB corneal allografts 7 days after the final AC injection. Results shown in Figure 5 demonstrate that three AC injections produced a significant prolongation in the survival time of grafts placed onto preimmune hosts.

DISCUSSION

The current results support previous findings indicating that the survival of orthotopic corneal allografts...
correlated with the acquisition of ACAID.11–13 The modest effect of AC priming with spleen cells in enhancing the survival of fully allogeneic corneal allografts is consistent with previous reports indicating that the induction of ACAID across MHC and multiple minor H barriers is transient.24 By contrast, AC priming with NZB spleen cells resulted in a remarkable reduction in the incidence and speed of rejection of minor H disparate corneal grafts. This is in keeping with earlier studies demonstrating that AC priming with minor H antigens elicits long-term suppression of cell-mediated alloimmunity.25 Results also demonstrate that Ia− nonadherent spleen cells are superior to Ia+ adherent cells in promoting corneal allograft survival. The disparity in the capacity of nonadherent and adherent lymphoid cells to promote corneal allograft survival might be expected because induction of ACAID against either the contact-sensitizing agent trinitrophenol or minor histocompatibility antigens requires the use of AC inocula containing Ia− nonadherent antigen-bearing cells.32,33

The failure of corneal epithelial cells to induce ACAID and to promote corneal allograft survival is surprising. However, the beneficial effects of AC priming with corneal endothelial cells in reducing the incidence of corneal allograft rejection supports previous observations correlating corneal allograft survival with the acquisition of ACAID.11–14 Moreover, the facile induction of ACAID with corneal endothelial cells and the failure to induce suppression of DTH with corneal epithelial cells further strengthen the hypothesis that corneal allograft survival is influenced significantly by ACAID, which is presumably induced by corneal endothelial cells shed from the graft.

Disruption of the camero-splenic axis by splenectomy has been shown to interfere with the induction of ACAID and, as reported here, results in a steep increase in the incidence and speed of the rejection of minor H disparate corneal allografts. The latter results support the notion that orthotopic corneal allografts induce ACAID, and maneuvers that interfere with graft-induced ACAID jeopardize corneal allograft survival.

Although corneal allografts enjoy a high rate of success, immunologic rejection remains the leading cause of corneal graft failure.1 The risk of rejection escalates in patients who already have rejected a corneal graft and presumably have been sensitized to donor alloantigens. It is noteworthy that ACAID can be induced to alloantigens in hosts extraocularly immunized with the same alloantigens.35 In the current study, we considered the prospect of inducing ACAID as a strategy for promoting corneal allograft survival in the presensitized host at high risk. As expected, the incidence and tempo of corneal graft rejection rose

### Table 1. Effect of Splenectomy on Corneal Allograft Survival

<table>
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<tr>
<th>Donor–Host</th>
<th>Graft</th>
<th>Manipulation</th>
<th>% Rejection</th>
<th>MST</th>
<th>P*</th>
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<td>Fully Allo</td>
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<tr>
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<td>Splx</td>
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<td>Splx</td>
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<td>80</td>
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<td>Splx</td>
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</table>

MST = mean survival time ± standard deviation.

*P* value determined by Mann-Whitney test.
sharply in hosts immunized with heterotopic corneal allografts before keratoplasty. However, AC priming with donor-specific alloantigenic cells produced a significant prolongation in graft survival in presensitized hosts at high risk.

The capacity of ACAID to promote corneal allograft survival in this model and others is impressive but does not guarantee 100% graft survival. It is unclear whether corneal graft rejection occurs solely by either DTH or CTL because there is compelling evidence to implicate both these cell-mediated immune effector mechanisms in corneal allograft rejection. In some rodent models of corneal graft rejection, indirect evidence suggests that DTH plays a dominant role. By contrast, other studies have correlated corneal allograft rejection with the development of allospecific CTL. It could be that corneal grafts, like other categories of organ grafts, are vulnerable to multiple rejection pathways because of the redundancy of the mammalian immune system. Even though ACAID promotes downregulation of allospecific DTH, CTL responses are spared. Thus, graft-induced CTL could account for the failure of ACAID to produce 100% graft acceptance in this and previous studies.

The facility with which corneal allografts, especially MHC-matched minor H-mismatched grafts, induce ACAID may help to explain the remarkable immunologic privilege of corneal allografts. However, it should be emphasized that a constellation of other features of the corneal graft and graft bed contribute to the success of corneal allografts. These include the avascular and alymphatic nature of the corneal graft bed, the absence or dearth of resident antigen-presenting cells (e.g., Langerhans cells) within the corneal graft, the elaboration of immunosuppressive factors by corneal cells, and the paucity of class I MHC antigens on the corneal endothelium. Optimizing and exploiting each of these parameters may markedly improve corneal allograft survival, especially in the patient at high risk.

Key Words

anterior chamber-associated immune deviation (ACAD), corneal allografts, immune privilege, keratoplasty

References


