Murine Orthotopic Corneal Transplantation in High-Risk Eyes
Rejection Is Dictated Primarily by Weak Rather Than Strong Alloantigens

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Purpose. Using a model of orthotopic corneal transplantation in which allografts were placed in normal eyes of mice, the authors previously reported that grafts bearing minor H antigens alone are more likely to be rejected (approximately 50%) than are grafts displaying only major histocompatibility (MHC) alloantigens (20%). These studies have been extended to include corneal grafts placed in neovascularized high-risk eyes of recipient mice.

Methods. Neovascularization was induced by placing sutures in the central cornea of one eye of recipient mice. Two weeks later, MHC class I only, class II only, minor H only, or MHC + minor H disparate corneas were grafted into these sutured eyes, and their rejection rates were examined.

Results. Although MHC + minor H disparate corneal allografts were rejected uniformly in neovascularized graft beds in 12 (100%) of 12, MHC class I only disparate grafts were rejected in 8 (66.7%) of 12 and MHC class II only disparate corneal allografts were rejected in 7 (58.3%) of 12. Surprisingly, the rejection rate of minor H only disparate corneal allografts was 10 (90.9%) of 11.

Conclusions. These findings indicate that for orthotopic corneal allografts placed in high-risk graft beds, minor H antigens offer a more formidable barrier to graft acceptance than do MHC-encoded antigens. The authors speculate that this unexpected outcome may reflect a reduced level of MHC expression on corneal tissue. Moreover, because the cornea as a graft lacks bone marrow-derived dendritic cells, allorecognition by recipient T cells must occur by way of the indirect pathway of alloantigen processing, and in this situation, minor H antigens may compete favorably with MHC antigens for presentation by recipient antigen-presenting cells that infiltrate the graft. Invest Ophthalmol Vis Sci. 1997;38:1130-1138.

In transplantation of histoincompatible tissues, the intensity of the immune rejection response, and the vigor with which the graft is destroyed depend on the strength of alloantigens that are expressed on the graft. In unmodified recipients, tissue transplants that express strong alloantigens encoded by loci within the major histocompatibility complex induce strong immunity, and these grafts are rejected promptly. By contrast, tissue transplants that confront their hosts with only minor histocompatibility antigens induce a less-vigorous immune response, and these grafts may experience prolonged survival before their eventual rejection.

In orthotopic corneal transplantation in humans, a surprisingly high proportion of grafts, especially in so-called low-risk situations, are successful, compared with other types of organ transplants. Moreover, the evidence concerning a role for antigens encoded by the human major histocompatibility complex (MHC), human leukocyte antigen (HLA), in corneal allografts is controversial. Some investigators claim that tissue typing, which permits cornea donor and recipient to be matched for HLA alloantigens, correlates with improved graft survival, whereas other investigators have been unable to detect such an effect. Experimental
We reported previously that sutured corneas possessed high-risk eyes alloantigens encoded by MHC loci are of Langerhans cells in the central cornea. Moreover, corneal neovascularization and a significant number with high-risk graft beds, we placed sutures in the central cornea of recipient mice 2 weeks before grafting. To examine corneal allograft rejection in eyes of mice of experiments, we wished to determine whether in low-risk eyes, these factors are believed to maintain immune privilege. In this situation, minor H alloantigens are more important than are MHC alloantigens for graft rejection. Although a high proportion of human orthotopic corneal allografts succeed, significant numbers of corneal allografts placed in so-called high-risk eyes fail. To examine corneal allograft rejection in eyes of mice with high-risk graft beds, we placed sutures in the central cornea of recipient mice 2 weeks before grafting. We reported previously that sutured corneas possessed corneal neovascularization and a significant number of Langerhans cells in the central cornea. Moreover, these eyes no longer support the induction of anterior chamber-associated immune deviation. In these high-risk graft beds, orthotopic corneal allografts that confront recipients with both MHC and minor alloantigens are rejected uniformly and swiftly, whereas only 50% of similar allografts placed in normal avascular (low-risk) graft beds are accepted indefinitely.

The high success of orthotopic corneal allografts in low-risk eyes is thought to result from the immune privilege of the eye, and in this situation, minor H alloantigens are more important than are MHC alloantigens for graft rejection. By contrast, high-risk eyes no longer maintain an intact immune privilege environment, and completely disparate corneal allografts are more susceptible to rejection. In the current series of experiments, we wished to determine whether in high-risk eyes alloantigens encoded by MHC loci are more important than are minor alloantigens for graft rejection.

**MATERIALS AND METHODS**

**Mice**

Cornea graft donors and recipients were adult mice (6 to 8 weeks of age) purchased from Taconic Farm (Germantown, NY) or Jackson Laboratories (Bar Harbor, ME). Donor and recipient pairs were selected to represent different levels of immunogenetic disparity, as presented in Table 1. The following inbred strains were used: BALB/c, C57BL/10, B10.D2, A.TL, A.TH, and A.SW. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of Corneal Neovascularization**

Three interrupted sutures (11-0 nylon, 50-μm-diameter needle, Sharpont; Vanguard, Houston, TX) were placed in the central cornea of one eye of normal BALB/c mice. As described previously, these sutures induce corneal neovessels from the limbus as early as 5 days; after 2 weeks, neovessels occupy more than two quadrants of the cornea. Mice with neovascularized graft beds then served as recipients of orthotopic corneal transplants.

**Corneal Transplantation and Grafting**

Orthotopic corneal transplantation was performed as described previously. Briefly, donor central corneas (2-mm diameter) were excised by vannas scissors and placed in chilled phosphate-buffered saline. Recipients were anesthetized with intraperitoneal injections of ketamine (3 to 4 mg/recipient) and xylazine (0.1 mg/recipient). The graft bed was prepared by excising with vannas scissors a 2-mm site in the central cornea of the right eye. The donor cornea then was placed in the recipient bed and secured with eight interrupted sutures (11-0 nylon). All grafted eyes were examined after 72 hours; at that time, grafts with technical difficulties (e.g., hyphema, infection, or loss of anterior chamber) were excluded from further consideration. At 9 days after grafting, all sutures were removed.

**Evaluation and Scoring of Orthotopic Cornea Transplants**

After corneal transplantation, grafts were examined by slit-lamp microscopy at weekly intervals. At each timepoint, grafts were scored for opacity and neovascularization. A scoring system was devised to describe in semiquantitative terms the extent of opacity (0 to 5+) as follows: 0 = clear graft; 1+ = minimal superficial (nonstromal) opacity; 2+ = minimal deep (stromal) opacity; 3+ = moderate superficial (nonstromal) opacity; 4+ = moderate deep (stromal) opacity; 5+ = severe superficial (nonstromal) opacity; 6+ = severe deep (stromal) opacity; 7+ = total rejection; 8+ = total rejection. All scoring was done by slit-lamp microscopy. Opacity was scored using a grading scale with five grades: 0 (clear), 1 (minimal), 2 (moderate), 3 (severe), and 4 (total). Neovascularization was scored using a grading scale with three grades: 0 (no neovascularization), 1 (neovascularization limited to the limbal region), and 2 (neovascularization extending to the corneal stroma).
TABLE 1. Immunogenetic Relationships of Cornea Graft Donors and Recipients

<table>
<thead>
<tr>
<th></th>
<th>Donor</th>
<th>Recipient</th>
<th>Type of Disparity</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Syngeneic</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>None</td>
</tr>
<tr>
<td>MHC + minor</td>
<td>C57BL/10</td>
<td>BALB/c</td>
<td>H-2, plus multiple minor H of C57BL/10</td>
</tr>
<tr>
<td>Class I only</td>
<td>A.SW</td>
<td>A.TH</td>
<td>H-2 Kα</td>
</tr>
<tr>
<td>Class II only</td>
<td>A.TL</td>
<td>A.TH</td>
<td>H-2 laβ</td>
</tr>
<tr>
<td>Minor H only</td>
<td>B10.D2</td>
<td>BALB/c</td>
<td>Multiple minor H of C57BL/10</td>
</tr>
</tbody>
</table>

MHC = major histocompatibility complex.

mal) opacity; 3+ = moderate stromal opacity; 4+ = intense stromal opacity; 5+ = maximum stromal opacity. Grafts with opacity scores of 2+ or greater at 8 weeks were considered to have been rejected.

Statistical Analyses

Corneal graft rejection was evaluated using a two-tailed Fisher's exact test. P < 0.05 was deemed significant.

RESULTS

Pattern of Survival of Syngeneic Corneal Grafts

To examine the fate of orthotopic syngeneic corneal transplants placed in graft beds that displayed suture-induced neovascularization, the first series of corneal transplants performed in BALB/c mice used syngeneic grafts. Corneal buttons harvested from normal eyes of BALB/c donors were grafted into eyes with corneal neovascularization, and the grafts were examined by slit-lamp microscopy and evaluated clinically using a scoring system described previously. The cumulative experience of scoring 10 such grafts for 8 weeks after transplantation is presented in Figure 1. After 1 week, all syngeneic grafts displayed moderate edema and opacity (1 to 3+) throughout the stroma. By the time of the second week after grafting, edema and opacity had begun to recede from some grafts. Three grafts of 10 showed minimal superficial opacity until 4 weeks after grafting, and at 8 weeks, no grafts except 1 displayed any evidence of edema or opacity. This one graft showed minimal superficial (but not stromal) opacity at 8 weeks. We previously reported that syngeneic grafts placed in normal graft beds experienced a similar postoperative course. Thus, syngeneic cornea grafts, which can elicit no alloimmune response, are well tolerated and survive indefinitely when placed in either normal eyes or in eyes with suture-induced neovascularization. This indicates that these abnormal corneal graft beds do not prejudice the survival of orthotopic grafts if the latter offers no immunogenetic barriers.

Pattern of Survival of Orthotopic Corneal Allografts Disparate at Major and Minor Histocompatibility Loci

Previously, we have shown that only 50% of corneal allografts that differed at both major and minor histo compatibility loci were rejected in normal avascular graft beds. The acceptance of orthotopic corneal allografts in normal graft beds can be ascribed to immune privilege. We wanted to examine whether orthotopic corneal allografts could be accepted in suture-induced neovascularized graft beds, where several aspects of immune privilege had been lost. Twelve BALB/c mice with neovascularized corneas received orthotopic corneal allografts from C57BL/10 donors. Criteria for rejection of cornea grafts were based on a scoring system described previously. Grafts achieving opacity scores of 2+ or greater were regarded as rejected. Using these criteria, patterns of graft survival were observed and are presented in Figure 2. At 1 week after grafting, the appearance of these allografts was virtually identical to that described previously for syngeneic grafts. However, by 2 weeks after grafting, all grafts displayed moderate-to-intense stromal opacity that never cleared, and these grafts were considered to have been rejected. Therefore, the incidence of rejection of corneal allografts that confront recipients with MHC plus multiple minor H alloantigens was 100%, and all grafts suffered rejection within 2 weeks after grafting. These results indicate that, unlike in normal recipient eyes, immune privilege for corneal allografts was not observed in suture-induced neovascularized eyes.

Pattern of Survival of Orthotopic Corneal Grafts Disparate Only at a Single Class I Major Histocompatibility Complex Locus

For other types of solid tissue transplants, such as skin, heart, and kidney, allografts displaying MHC disparities are rejected uniformly. Because it has been shown that corneal tissue expresses reduced amounts of MHC class I and no class II molecules, the next series of experiments was designed to examine the
Corneal Transplantation in High-Risk Eyes

Opacity Score

Syngeneic corneal grafts

Weeks after grafting

FIGURE 1. Survival patterns of syngeneic orthotopic corneal grafts: BALB/c donors–BALB/c recipients (number = 10). Weekly opacity scores based on clinical examinations of grafts of individual animals are displayed for an 8-week observation period. Basis for arriving at scores is described in Materials and Methods.

fate of MHC class I only disparate corneal allografts in neovascularized graft beds and used A.TH mice. The BALB/c mice, used in the previous experiments, and A.TH mice, used in these experiments, have a comparable ability to mount rejection of allografts as shown by equivalent rejection of allogeneic skin grafts. Twelve A.TH mice with corneal neovascularization received transplants from A.SW donors. In this instance, the only alloantigen expressed by the graft is encoded by $H-2^K$. Figure 3 presents a summary of the clinical

Opacity Score

MHC + Minor H disparate corneal allografts

Weeks after grafting

FIGURE 2. Survival patterns of major histocompatibility plus minor H incompatible orthotopic corneal grafts: C57BL/10 donors–BALB/c recipients (n = 12). Results of individual grafts are displayed as described in Figure 1. (+) indicates grafts with irreversible rejection (n = 12, 100%).
course of these transplants. All grafts experienced acute inflammation at 1 to 2 weeks after grafting; however, 4 of 12 corneal allografts displayed diminished opacity at 3 weeks and remained clear until 8 weeks. Therefore, the incidence of rejection of class I only disparate corneal allografts in neovascularized graft beds was estimated at 8 (66.7%) of 12, which is significantly less than that of MHC + minor H disparate corneal allografts.

**Pattern of Survival of Orthotopic Corneal Grafts Disparate Only at Class II Major Histocompatibility Complex Loci**

To examine the fate of MHC class II only disparate corneal allografts in neovascularized graft beds, 12 A.TH mice with corneal neovascularization received orthotopic corneal allografts from A.TL donors. As the results summarized in Figure 4 show, moderate opacity developed in all class II disparate only grafts at 1 to 2 weeks after grafting. However, only 7 (58.3%) of 12 of these allografts suffered from irreversible rejection, whereas five allografts showed no or minimal opacity at 8 weeks after grafting and were considered to have been accepted. The tempo of graft rejection was slower than that of MHC + minor H or MHC class I only disparate corneal allografts, because the majority of failed grafts was detected at 3 to 4 weeks after grafting.

**Pattern of Survival of Orthotopic Corneal Grafts Disparate Only at Minor Histocompatibility Loci**

Because all orthotopic corneal allografts that differed at both MHC + multiple minor H loci form recipients were rejected, whereas only 50% to 60% of MHC class I only, or class II only disparate orthotopic corneal allografts were rejected in neovascularized graft beds, the following experiments were performed to examine the fate of minor H only disparate corneal allografts in neovascularized graft beds. B10.D2 corneas were grafted to the neovascularized corneal graft beds of BALB/c mice. Figure 5 summarizes the results of 11 minor histocompatible only transplants. After an initial inflammation that was similar to that found in other transplants, all minor H only incompatible grafts, except one, displayed moderate-to-intense opacity within 2 weeks after grafting and remained opaque at 8 weeks. Only 1 (9.1%) of 11 grafts diminished its opacity around 5 weeks and became clear at 8 weeks. The incidence of rejection of minor H only disparate corneal allografts was 10 (90.9%) of 11, which was as high as that found when grafts displaying the greatest degree of immunogenetic disparity are examined.

The results of this series of experiments are summarized in Figure 6 and Table 2. These results, along with our previous findings, indicate that the transplantation antigens on cornea grafts that are most effective at inducing irreversible rejection, whether in avascular low-risk graft beds or in neovascularized high-risk graft beds, are encoded by minor histocompatibility loci, not class I or class II antigens encoded within the MHC.

**DISCUSSION**

Even though it is well known that corneal transplantation is the most successful of solid organ transplants...
in humans, significant numbers of corneal allografts placed in high-risk eyes fail. Unlike other types of organ transplantation, it has not been easy to show that the degree of immunogenetic disparity between donor and recipient is an important factor in high-risk situations. Although several studies have reported that HLA matching, especially for class I alloantigens, improves the fate of corneal allografts in humans,6,27,28 other studies claim that there is no discernible effect of HLA matching.5,7,29 A recent multicenter collaborative study (the Collaborative Corneal Transplantation Studies) completed in the United States came to the
conclusion that HLA matching, whether for class I or class II alloantigens, has no influence on corneal graft outcome in high-risk eyes, whether assessed as rejection reaction, rejection, or all-cause graft failure. If HLA disparity is not the dominant factor in corneal allograft rejection, then minor histocompatibility antigens must be rather important. To that end, the studies described in this communication show that minor only disparate corneal allografts were rejected more frequently than were MHC class I or class II only disparate corneal allografts in high-risk neovascularized graft beds of mice. These results indicate that the transplantation antigens on corneal grafts that are most effective at inducing irreversible rejection in mice are encoded by minor histocompatibility loci, not class I or class II antigens encoded within the MHC.

The reasons why minor H only disparate corneal allografts are rejected more readily than are MHC class I or class II only disparate corneal allografts are obscure. In other types of organ transplantation, such as skin, kidney, heart, and liver, transplants that express strong alloantigens encoded by MHC induce strong immunity, and these grafts are rejected uniformly. In these cases, the grafts routinely contain so-called passenger leukocytes (e.g., Langerhans cells and dermal dendritic cells in the skin, Kupffer cells in the liver), which express high levels of MHC class II molecules, are highly mobile, and sensitize recipient T cells by migrating from the graft to the draining lymph nodes. This pathway of allore cognition has been called the direct pathway, because recipient T cells recognize donor MHC alloantigens directly on donor-derived cells. Normal corneas contain virtually no passenger leukocytes—neither Langerhans cells in the epithelium, nor dendritic cells or macrophages in the stroma. Thus, corneal allograft recipients are not sensitized by this direct pathway. Sensitization of recipients must occur by way of an indirect pathway at which recipient APCs infiltrate the graft, acquire donor antigens, and present them to T cells. It has been shown that both MHC-encoded alloantigens and minor H alloantigens can be recognized by way of this indirect pathway. In this pathway, peptides derived from these antigens are loaded onto class II molecules of recipient APC and presented to T cells. Because corneal tissue is reported to have reduced expression of class I and no class II MHC antigens, peptides from these alloantigens will not compete with peptides from minor H alloantigens for presentation by recipient APC. This may allow corneal cells to present more minor H type proteins than MHC-encoded molecules. Therefore, minor H antigens may be the major source of corneal peptides that can be recognized by way of the indirect pathway. In fact, we previously reported that mice that reject grafts placed in either normal avascular graft beds or in neovascularized graft beds acquire donor-specific DH directed at minor, rather than MHC, alloantigens.

In the clinic, recipient eyes can be regarded as high risk for many reasons: stromal vascularization, history of previous graft rejection, uncontrolled glaucoma, decreased corneal sensation, presence of persistent active intraocular inflammation, and associated ocular abnormalities, such as eyelid disease, abnormal conjunctiva, or dry eye syndromes. Several features observed in murine eyes with suture-induced corneal neovascularization used in this study can be considered as contributing to the high-risk situation. First, the sutured-cornea contains blood vessels, which can allow immune effector cells and molecules to gain access into the graft and to destroy the graft tissue. Second, suture-induced corneal neovascularization in mice is accompanied by Langerhans cell migration into the central corneal epithelium. Because donor corneal tissue contains no indigenous donor-derived APCs, recipient APCs may prove to be important for sensitization of recipient T cells to donor alloantigens. In normal corneal beds, recipient APCs exist only in the limbus of the corneal epithelium. We previously

### TABLE 2. Summary of Fate of Orthotopic Corneal Allografts

<table>
<thead>
<tr>
<th>Type of Disparity</th>
<th>Number</th>
<th>Accepted Grafts</th>
<th>Rejected Grafts</th>
<th>Rejection Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngeneic</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MHC + minor</td>
<td>12</td>
<td>0</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Class I only</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>66.7*</td>
</tr>
<tr>
<td>Class II only</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>58.3*</td>
</tr>
<tr>
<td>Minor H only</td>
<td>11</td>
<td>1</td>
<td>10</td>
<td>90.9</td>
</tr>
</tbody>
</table>

MHC = major histocompatibility complex.

* Rejection rate is significantly lower than that of MHC + minor disparate grafts (two-tailed Fisher's exact test) \( P < 0.05 \).
reported that eyes with suture-induced corneal neovascularization contain significant numbers of Langerhans cells in the central corneal epithelium. We proposed that in these eyes, recipient corneal beds already contain significant numbers of recipient APCs at the time the corneal allografts are transplanted, and that these APCs migrate into the graft more rapidly than when grafts are placed in normal corneal beds. We have examined the frequency of Ia+ mononuclear and dendritic cells within the central epithelium of C57BL/6 grafts placed in either normal or neovascularized graft beds of BALB/c mice. Significantly greater numbers of Ia+ dendritic cells were found in neovascularized graft beds as compared with those of normal beds 7 to 28 days after grafting (Sano and Streilein, unpublished data, 1995). Therefore, one reason why corneal allografts placed in neovascularized graft beds are rejected more frequently may be because of the existence of recipient APCs in the graft beds, cells that can migrate into the graft beds immediately after transplantation. In fact, Williams et al. have shown that patients whose corneas contained <50 HLA-positive cells/mm² experienced a 3-year actuarial graft survival of 83%, compared with 39% in those whose corneas contained >50 such cells/mm². Although APCs can migrate into the grafts, it is unclear how APCs might escape from the cornea and present reprocessed allantogens to recipient T cells. Collin reported the existence of lymphatic vessels in neovascularized rabbit corneas. Therefore, we also suggest, but have no evidence, that murine corneas with suture-induced neovascularization possess lymphatic vessels through which APCs carry antigens to the local draining lymph nodes.

When our results are considered in light of our previous findings, we conclude that the rejection of orthotopic corneal allografts placed in either normal avascular graft beds or neovascularized high-risk graft beds is directed at minor H, rather than MHC, allantogens. Because donor corneal tissue is devoid of donor-derived APCs, our findings imply that recipient APCs must play a central role in the process by which T cells recognize allantogens and become sensitized. Thus, the existence of APCs in the central cornea of the recipient eyes can be considered to be one of the most important factors that render eyes high risk.

Key Words
corneal transplantation, high-risk eyes, major histocompatibility complex, minor H, mouse

References
20. Sano Y, Streilein JW. Effect of suture-induced corneal


