Effect of Mismatches for Major Histocompatibility Complex and Minor Antigens on Corneal Graft Rejection

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The importance of minor histocompatibility genes in corneal graft rejection was investigated using a model that simulates the major histocompatibility complex (MHC) and minor mismatches of the human allograft more accurately than previous animal models. DA(RT1a) X LEW(RT1')F₁ hybrid rats were backcrossed to LEW, and the backcross generation were used as corneal graft recipients. Female DA(RT1a) strain animals were used as donors throughout. As in humans, the MHC disparity (a to 1) between each donor-recipient pair could be controlled; minor mismatches were variable and unknown. The MHC haplotype of each backcross individual (either homozygous 1/1) or heterozygous a/1) was determined. Depending on this haplotype, the transplanted DA cornea was either matched or mismatched with the recipient for MHC antigens. The average proportion of minor disparate loci was 50%, although this was variable and unknown from recipient to recipient. Some animals of each MHC type were sensitized with three subcutaneous DA strain skin grafts at intervals of 2 weeks. Prior sensitization caused more rapid corneal graft rejection in both MHC mismatched (P < 0.001) and matched (P < 0.01) animals. All animals in the two MHC-mismatched groups (sensitized, 26; unsensitized, 17) and most in the MHC-matched groups (sensitized, 25 of 27; unsensitized, all 13) rejected their grafts. The MHC matching resulted in a greater range of survival times, although the difference in survival in unsensitized animals between matched and mismatched groups was not significant (unsensitized, P > 0.05; sensitized, P < 0.001). Thus, minor antigens played a significant role in corneal graft rejection in this rat model, and the high rejection rate of MHC-matched grafts suggested that, as with skin, several minor genes were involved. If such genes also are important in humans, matching for MHC in high-risk cases may be of limited value. Invest Ophthalmol Vis Sci 32:2729-2734, 1991

Evidence from different investigations concerning the value of matching in corneal transplantation is conflicting, although the balance of recent evidence, involving prospective studies, suggests that histocompatibility antigen HLA-A, -B, and -DR matching is beneficial to long-term graft survival. The delay in establishing the value of matching is partly a result of the relative immunologic privilege of the cornea afforded by the natural absence of blood and lymphatic vessels. However, rejection episodes frequently occur in humans, especially if the graft bed has become vascularized, and the ultimate high success rate (up to 90%) of a first corneal graft may be related to the exposed location of the eye. This enables direct application and penetration of immunosuppressive drugs, and early signs of rejection can be seen easily. These fortunate characteristics may have disguised the cornea's underlying potential for rejection. Thus, matching for major histocompatibility complex (MHC) antigens was not considered to be necessary, even in "high-risk" cases in which the eye was vascularized or a previous graft failed, and the role played by minor antigens in rejection was neglected.

In rat models where no immunosuppression is given and where corneal vascularization occurs more readily than in humans, the incidence of rejection can reach 100%, especially if recipients respond vigorously to MHC antigens. Recently it was shown in the rat that minor antigens may play an important role in rejection. In this study, using our high-responder rat model, in which recipients are hybrid rather than inbred, we independently confirm and extend these observations in both sensitized and unsensitized animals. The clinical characteristics of rejection in the model also are described. Use of hybrid recipients has two advantages over other models: (1) it simulates the
human situation whereby MHC mismatches for each donor–recipient pair can be controlled, but minor disparity in the population is variable and unknown for each individual, and (2) it has enabled us to conclude that several independently segregating minor genes are involved in corneal graft rejection.

Materials and Methods

Rats

DA(RT1a), LEW(RT1b), and (DA X LEW) F1 hybrid rats were purchased from Harlan Olac (Bicester, United Kingdom). The DA and LEW strains differ across the entire MHC and at an unknown number of minor histocompatibility loci. The (DA X LEW)F1 hybrids were backcrossed to LEW in our laboratory to provide recipients for female DA strain corneal grafts. All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Experimental Groups

We divided (DA X LEW)F1 X LEW backcross generation rats into two groups on the basis of tissue type, i.e., heterozygous RT1a/1 or homozygous RT1b/1. Animals of both types were used as recipients for DA (RT1a/a) strain corneal grafts (Fig. 1). Thus, donor and recipient were either matched or mismatched for MHC antigens. Some animals in each group were sensitized to RT1a antigens by DA strain skin grafts. Equal proportions of male and female animals were assigned to each group. Control groups consisted of syngeneic grafts (LEW to LEW and DA to DA) and parental to F1 (DA to [DA X LEW]). Eighteen syngeneic recipients were female, and two were male.

Tissue Typing

Rat erythrocytes express class I (RT1A) antigens. Therefore the RT1a haplotype was identified by indirect hemagglutination using a monoclonal antibody, MN4-91-6, against RT1Aa antigen.11 This assay gives unequivocal results. Blood was collected into anticoagulant (Alsever’s solution, pH 6.1) by cardiac puncture under anesthesia. After three washes in cold phosphate-buffered saline (PBS), packed red cells were diluted 1:100 in PBS containing 2% fetal calf serum. The MN4-91-6 antibody, supplied as purified ascites fluid, was diluted 1:500 and placed in 25-μl volumes in wells of a nonsterile 96-well microtiter plate. We added 25 μl of the erythrocyte suspension to the monoclonal antibody preparation. The mixture was incubated at 4°C for 45 min; then the cells were washed three times in PBS containing 2% fetal calf serum. In addition, 50 μl of a 1:500 dilution of sheep anti-mouse immunoglobulin G (Serotec, Oxford, United Kingdom), which had been preabsorbed with rat erythrocytes, was added to each well. The plates were covered with a sticky cover (Titertek; ICN Flow, Rickmansworth, United Kingdom), agitated on a vortex machine to resuspend the cells, and allowed to stand at 4°C for approximately 1 hr. Cells expressing the RT1Aa antigen agglutinated; those of the homozygous RT1A1 type did not.

Skin Sensitization

Donor animals were killed and shaved on both sides of the trunk. The skin was swabbed with alcohol, removed, and pinned onto a cork board, and muscle and connective tissue were scraped off with a sterile scalpel blade. Squares approximately 1.5 X 1.5 cm in size were cut and stored until required (up to 3 hr) on filter paper soaked in sterile saline at 4°C. The recipient was anesthetized by intramuscular injection of 0.5 ml/kg fentanyl-fluanisone (Hypnorm; Janssen, Oxford, United Kingdom) in one hind limb and 2.5 mg/kg diazepam (Valium; Roche, Welwyn Garden City,
United Kingdom) in the other. A subcutaneous pocket was made to one side of the ventral midline of the thorax, and the donor skin square was placed flat, outer surface uppermost, in the pocket. The wound was closed with four or five 4–0 silk sutures (Ethicon, Edinburgh, United Kingdom), dusted with neomycin powder (Cicatrin; Wellcome, London, United Kingdom), and covered with sterile gauze. The gauze was secured with surgical tape wrapped around the body (Blenderm; 3M, St. Paul, MN); this was removed after 2–3 days. The first skin graft was done when the animals were 8–9 weeks of age. Subsequent skin grafts were sited immediately posterior to the previous graft 2 weeks after the graft.

**Corneal Grafts**

Unsensitized animals were 12–22 weeks of age when they received a corneal graft. Sensitized animals were 15–16 weeks of age when they received their corneal grafts; this was 19–22 days after they received the last skin graft. Corneal grafting was done on the right eye as previously described, except that the rats were anesthetized as mentioned with Hypnorm and Valium, rather than with halothane. The donor cornea was secured with eight interrupted 10–0 nylon sutures, which were not removed. We applied one drop of atropine sulfate 1% and chloramphenicol ointment 1% to these eyes immediately postoperatively and on days 1, 2, 3, 5, and 7.

**Clinical Examination**

The grafts were examined daily for 3 days after surgery using a slit lamp and then four times weekly until rejection occurred or until day 50. Thereafter, they were examined twice weekly. Donor corneas were graded for central corneal opacity (1–4), edema (1–3), and vessel ingress (1–4, with 1 denoting vessels just beyond the graft margin and 4, vessels to the center).

The day of rejection was defined as the day when central donor corneal opacity and edema became moderate or severe (ie, a score of 2 or more); this persisted for several days or became worse. Clinical signs of rejection were confirmed by histologic examination of a randomly chosen sample of corneas. The grafts were considered to be accepted if they had not been rejected by postoperative day 100.

**Results**

**The Corneal Grafting Model**

Of 110 grafts done, seven were technical failures because the anterior chamber did not reform or because of iris prolapse. Syngeneic grafts (LEW to LEW \([n = 7]\) and DA to DA \([n = 5]\)) or parental to F1 hybrid (DA to [DA × LEW] \([n = 8]\)) became clear in 7–14 days and remained clear for at least 100 days. Maximum opacity of such grafts (usually during the first week) was less severe than that seen in rejected grafts. Rejection was observed as increasing corneal opacity progressing from the graft margin and beginning either unilaterally (where vessels were most prominent) or uniformly from all directions. This was accompanied by edema and a heavy cell infiltrate into the stroma consisting of neutrophils and mononuclear cells. An endothelial rejection line, as seen in humans, was not identified, although there was histologic evidence of endothelial cell destruction (absence of cells and/or inflammatory cells adhering to the endothelium). In all sensitized animals that rejected their grafts and in 22 of 30 (73%) unsensitized animals, corneal opacity reached a score of 3 (the pupil was barely visible through the cornea) or more (Fig. 2). Vessels reached the sutures approximately 1 week postoperatively. In corneas receiving syngeneic grafts and in allografts that were not rejected, vessels did not progress far beyond the stitches (Fig. 3). However, corneas undergoing rejection became severely vascu-
larized, often to the center. In unsensitized animals, signs of rejection did not occur until vessels reached the graft margin. Recipient corneas were relatively clear when the rejected donor corneas became opaque (Fig. 2). However, in sensitized animals, rejection frequently occurred before the vessels reached the graft margin, and in these instances, the recipient cornea also became opaque, presumably because lymphocytes passed directly through the stroma, rather than within the vessels, into the graft.

Twenty-five unsensitized animals were examined for a prolonged period (up to 40 days) after rejection. Twenty-three of these showed improvement in corneal clarity over a period of about 3 weeks from very severe or severe to moderate or mild opacity. This appeared to correlate with the reestablishment of an endothelial cell layer, seen both on histologic sections and on silver-stained endothelial flat-mount preparations. There was no difference in graft survival times between male and female recipients.

**Rejection in Animals With Differing Degrees of Donor–Recipient Mismatch**

All allografts, whether matched or mismatched for MHC antigens, were rejected, except in the case of two animals in the sensitized MHC-matched group (Table 1). The grafts were rejected significantly more rapidly in animals presensitized to the donor. In sensitized animals, MHC-mismatched grafts were rejected significantly more rapidly than those that were matched. However, although grafts in unsensitized animals were rejected more rapidly if they were MHC mismatched, the difference was not significant. There was no apparent difference between matched and mismatched grafts in the clinical signs of rejection; the extent of vascularization and the histologic appearance of corneas were similar in each group.

**Discussion**

The routine use of immunosuppressive therapy makes it difficult to assess the underlying potential of the human cornea for rejection. However, it is important to be fully aware of this potential to achieve the best drug and tissue-matching protocols. Therefore, for our investigation, we tried to promote rejection. This was done by producing hybrid recipients from a strain (LEW[RT1']) that shows a high response to alloantigens and by encouraging corneal vascularization by using interrupted sutures that were not removed. We observed a high incidence of rejection, even in recipients that were matched with the donor for MHC and not presensitized to donor antigens. Other workers have shown that the incidence of rejection of MHC-mismatched grafts can be high even when vascularization is minimized, either by use of a continuous suture or by removal of sutures soon after surgery. This suggests, on superficial consideration, that the rat is not a good model for human rejection. However, if we omitted immunosuppressive therapy in humans and took account of our longer life span, the incidence of rejection over a comparable period might approach that seen in the rat.

The resolution of rejection we observed in our model also was reported by others. A likely explanation for this lies in the capacity of rat endothelial cells to divide after injury. Others found that if the endothelium was removed from a 3-mm autograft button before it was sutured back into place, a combination of mitosis and cell sliding restored a partial monolayer after 48 hr. The endothelial cells achieved pregraft

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**Table 1. Corneal graft survival time in donor–recipient pairs either matched or mismatched for MHC antigens**

<table>
<thead>
<tr>
<th>Recipient presensitized to DA*</th>
<th>MHC (donor recipient)</th>
<th>MHC MM</th>
<th>Minor MM</th>
<th>No. of animals</th>
<th>Corneal graft survival time (days)</th>
<th>Median survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>RT1&lt;sup&gt;aa&lt;/sup&gt; → RT1&lt;sup&gt;1/1&lt;/sup&gt;</td>
<td>a</td>
<td>Yes</td>
<td>17</td>
<td>7, 8, 9, 9, 9, 10, 10, 11, 11, 11, 11, 13, 13, 14, 15</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Yes</td>
<td>RT1&lt;sup&gt;aa&lt;/sup&gt; → RT1&lt;sup&gt;1/1&lt;/sup&gt;</td>
<td>a</td>
<td>Yes</td>
<td>26</td>
<td>4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 6, 6, 6, 6, 6, 7</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>RT1&lt;sup&gt;aa&lt;/sup&gt; → RT1&lt;sup&gt;1/1&lt;/sup&gt;</td>
<td>—</td>
<td>Yes</td>
<td>13</td>
<td>7, 8, 9, 10, 11, 11, 11, 12, 12, 16, 17, 17, 18, 45</td>
<td>12&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Yes</td>
<td>RT1&lt;sup&gt;aa&lt;/sup&gt; → RT1&lt;sup&gt;1/1&lt;/sup&gt;</td>
<td>—</td>
<td>Yes</td>
<td>27</td>
<td>5, 5, 5, 6, 6, 7, 7, 7, 7, 8, 8, 8, 8, 8, 8, 9, 9, 9, 10, 10, 11, 12, &gt;100, &gt;100</td>
<td>8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
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Statistical analysis by Mann Whitney U test: *v<sup>b</sup> — P < 0.001, *v<sup>c</sup> — P < 0.01, *v<sup>d</sup> — P > 0.05, *v<sup>e</sup> — P < 0.001.

That is, to RT1<sup>a</sup> antigens plus all DA minor antigens.
density 14 days postoperatively, and normal corneal thickness was restored after 21 days. Thus, damage to the endothelium, caused either at the time of grafting or as a result of endothelial rejection, can be repaired by proliferation and migration of recipient cells on to the graft. Silver staining of the endothelium at various stages after rejection in our animals indicated that clearing of the graft was accompanied by restoration of the endothelial cell layer (data not shown). We did not observe a continuous fibrous retrocorneal membrane as seen in grafts from Wistar-Furth to Lewis rats.16

Although endothelial rejection may be an important component of rejection in the rat, the heavy infiltration into the stroma indicates that stromal cells are also a target. Again, direct comparisons with humans are difficult because immunosuppression reduces stromal rejection reactions in humans. Although rejection in unsensitized animals was never seen until vessels had reached the graft margin, in presensitized animals, it frequently occurred before the vessels had time to reach the graft. This showed that, as in humans, the vessels were not essential to convey the cells mediating rejection into the graft.

The high incidence of rejection of MHC-matched grafts was unexpected, particularly because the average minor mismatch was only 50%. These results parallel those seen with skin, in which multiple minor disparities in MHC-matched grafts can cause rejection as severe as that seen with MHC disparity alone.17 That skin was able to sensitize the donor strongly to an MHC-compatible corneal graft shows that skin and cornea possess common minor antigens. In view of their similar origins in the embryo, such shared antigens may be exclusive to skin and cornea.

The choice of the high-responder LEW strain in our crosses might account for the high incidence and rate of rejection of MHC-mismatched grafts. However, the RT1\r genes would not necessarily be expected to elicit a uniform high response to minor antigens. There may be a particularly large number of minor mismatches between DA and LEW strains or there may be genes controlling the immune response to corneal grafts that are not MHC linked.18 An alternative explanation is related to the current concept of recognition of foreign antigens by T-cells.19,20 This maintains that the amino acid sequences of the polymorphic parts of the MHC molecule determine the affinity with which a particular foreign (or self) peptide binds to the cleft of the molecule. Thus each allelotype binds a different repertoire of peptides, and the immune response to any given antigen will vary according to the host MHC allelotype. Heterozygosity for class II MHC on antigen-presenting cells permits presentation of a greater variety of peptides to helper T-cells. Therefore, the number of helper T-cell clones stimulated to react to minor antigens (presented as peptide in the recipient class II MHC cleft) is greater. The fact that our MHC-mismatched recipients were of the heterozygous RT1\r haplotype may thus have resulted in a stronger helper T-cell response. This may have contributed to the high level of rejection of MHC-matched grafts, and is similar to the human situation, where most individuals are polymorphic for MHC loci.

Because of the high rate of rejection (95%) in the MHC-matched groups, despite an average minor antigen disparity of only 50%, and assuming that disparity at a single locus is sufficient to cause rejection, we concluded that several independently segregating minor genes were involved in rejection in this rat model. These findings confirm the suggestion of others10 that, although matching for MHC is probably beneficial, it may not be sufficient to prevent rejection. The finding that minor antigens are important targets for rejection may help to explain why the value of MHC matching in humans has not always been apparent.

Key words: cornea, graft rejection, histocompatibility antigens, minor antigens, rat

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References