Characteristics of Corneal Xenograft Rejection in a Discordant Species Combination

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Purpose. To characterize the fate of Lewis rat corneas transplanted to Hartley guinea pigs.

Methods. Full-thickness Lewis rat corneal buttons were grafted orthotopically to Hartley guinea pigs (xenografts), ACI rats (allografts), or Lewis rats (isografts). Two panels of recipients were presensitized with xenogeneic skin grafts or allogeneic skin grafts. Serum samples were collected pre- and post-transplant and analyzed by flow cytometry and indirect immunofluorescence.

Results. Unlike vascularized xenografts that reject within 30 min, corneal xenografts had a mean survival time of 8 days. Presensitization with guinea pig skin grafts increased recipient IgM and IgG xenoantibody levels, as measured by flow cytometry on guinea pig hematopoietic cells, and significantly accelerated corneal xenograft rejection with a mean survival time of 5 days. Presensitization with allogeneic ACI skin grafts had no effect on xenoantibody levels or xenogeneic corneal graft survival. Guinea pig corneas stained by indirect immunofluorescence with normal rat serum exhibited low (1+) but significant binding of IgG and IgM, primarily on epithelium and stroma. Serum from Lewis rats that rejected a corneal xenograft had elevated IgG and IgM xenoantibodies that reacted strongly (4+) with guinea pig cornea and heart.

Conclusions. In the discordant guinea pig-to-rat species combination, donor corneas express xenoantigens; rejection of corneal xenografts stimulates IgM and IgG xenoantibody production; sensitization to xenoantigens can accelerate corneal xenograft rejection; and discordant corneal xenografts, unlike vascularized organs, are not hyperacutely rejected. Invest Ophthalmol Vis Sci. 1993:34:2469-2476.
In avascular graft site, the role of these humoral rejection mechanisms may be considerably diminished. Therefore, the transplantation of xenogeneic corneas may prove to be more successful than that of vascularized organs.

Worldwide, the shortage of donor corneas has been estimated to be as high as 10 million. The lack of corneas stems from many factors, including religious or ethnic beliefs, lack of adequate eye banking facilities, and poor quality of locally donated corneas. In the U.S., the need for corneas is not as acute, but availability varies from region to region. Potentially, as with other tissues and organs, the use of xenogeneic donors may be a way to alleviate the shortage of donor corneas.

Many early studies of corneal xenografts examined the survival of heterotopic9 or intralamellar grafts,10-12 which have only limited clinical use and are not subjected to the same exposure to the host immune system as are full-thickness grafts. Only a few investigators have used a penetrating keratoplasty technique to perform xenografts (Table 1).13-21 Of these, none have used animal species associated with well-established models of xenotransplantation. Concordant models of corneal xenotransplantation have yielded promising results18-21 but, as mentioned, are of little practical usefulness for human corneal transplantation.

We have examined the survival of guinea pig corneas transplanted to Lewis rat recipients. This discordant species combination has been widely used to examine the hyperacute rejection of vascularized xenogeneic transplants.1,2,8,22 Our findings indicate that, in contrast to cardiac xenografts, which fail in 17 ± 4 min,22 guinea pig corneal xenografts are not hyperacutely rejected in Lewis rats, and that rejection is significantly accelerated when the recipients are presensitized with xenogenic (but not allogeneic) skin grafts. Antibody labeling studies demonstrate that normal rat serum contains NAb that binds to antigens expressed on guinea pig cornea and that serum from rats immuno-

ized by corneal xenografts has elevated levels of IgM and IgG xenoantibody.

**METHODS**

**Animals**

All animals involved in this study were cared for and used in accordance with the ARVO Resolution on Use of Animals in Research. Male Lewis (RT1) rats and Hartley guinea pigs were obtained from Charles Rivers Laboratories (Wilmington, MA). ACI (RT1) rats were bred at the VA Medical Center Animal Resource Facility.

**Orthotopic Corneal Transplantation**

Full-thickness, 3.5-mm penetrating corneal grafts were performed as previously described.23 Briefly, animals were anesthetized by intramuscular injection of with a combination of 100 mg/kg of a 100 mg/ml solution of ketamine (Parke-Davis, Morris Plains, NJ) and 2 mg/kg of a 100 mg/ml solution of xylazine (Mebion, Shawnee, KS). Topical anesthesia, proparacaine (Alcon, Ft. Worth, TX) was applied to the eye. A subconjunctival injection of 0.05 ml of a solution containing 1 mg/ml atropine (Elkins-Sinn, Cherry Hill, NJ) and 1:1000 epinephrine (Parke-Davis) was used to dilate the iris. A 3.5-mm trephine was used to score the donor cornea, and the donor graft was removed using curved corneal scissors. The recipient cornea was similarly scored with a 3.0-mm trephine, and the central 3-mm area was removed. The donor graft was then sewn in place using 12 interrupted sutures of 11-0 nylon (Alcon). The anterior chambers of recipients were re-formed with sodium hyaluronate (Healon, Pharmacia, Piscataway, NJ). Six sutures were removed on postgraft day 5 and the remaining six on postgraft day 8. Topical antibiotic, tobramycin (Alcon), was applied immediately after surgery and on postoperative days 4 and 7. No immunosuppressant agents were administered at any time during the study.

**TABLE I. Historical Development of Orthotopic Corneal Xenotransplantation**

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Model</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kissam</td>
<td>1844</td>
<td>Pig to human</td>
<td>Failed</td>
<td>13</td>
</tr>
<tr>
<td>D'Amico</td>
<td>1969</td>
<td>Cat to rabbit</td>
<td>33% successful</td>
<td>14</td>
</tr>
<tr>
<td>Haq</td>
<td>1972</td>
<td>Fish to human</td>
<td>Failed</td>
<td>15</td>
</tr>
<tr>
<td>Durrani</td>
<td>1974</td>
<td>Bovine to human</td>
<td>21% improved vision</td>
<td>16</td>
</tr>
<tr>
<td>Bahn</td>
<td>1982</td>
<td>Bovine to cat</td>
<td>Failed</td>
<td>17</td>
</tr>
<tr>
<td>Moore</td>
<td>1987</td>
<td>Cat to rabbit</td>
<td>Failed</td>
<td>18</td>
</tr>
<tr>
<td>Insler</td>
<td>1991</td>
<td>Human to primate</td>
<td>67% successful</td>
<td>19</td>
</tr>
<tr>
<td>Li</td>
<td>1992</td>
<td>Human to primate</td>
<td>55% successful</td>
<td>20</td>
</tr>
<tr>
<td>She</td>
<td>1992</td>
<td>Mouse to rat</td>
<td>Successful</td>
<td>21</td>
</tr>
</tbody>
</table>
Grafts were observed every other day for the first week and at least twice a week thereafter. Grafts were graded for clarity, edema, and vascularization. When at least two of the three criteria were moderate to severe, the grafts were considered to be rejected.

Skin Grafts

Recipients were grafted orthotopically with 1 cm² donor skin on the thorax, as described. Grafts were inspected visually, and the percent of visible epithelial necrosis was recorded.

Measurement of Xenoreactive Antibody

Guinea pig cervical lymph nodes were removed and teased apart in phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma, St. Louis, MO) and 0.02% NaN₃ (PBA). Cells were then washed three times in PBA and resuspended at a concentration of 3 X 10⁶ cells/ml. Cells were stained with serial dilutions of serum using PBA as the diluent for 30 min at 4°C. After washing twice with PBA, cells were stained with a 1:100 dilution of goat anti-rat IgM-phycoerythrin conjugate and goat anti-rat IgG-fluorescein conjugate (Jackson Immunoresearch, West Grove, PA) for 30 min at 4°C. The cells were washed twice, fixed in 1% neutral buffered formalin, and analyzed on a FACScan (Becton-Dickinson, Mountain View, CA). The use of paired secondary antibodies allowed simultaneous assessment of rat IgG (FITC channel) and IgM (PE channel). Data are reported as mode fluorescence channel.

Immunofluorescence Histology

Guinea pig eyes or heart were embedded in gelatin and snap-frozen in a liquid nitrogen-immersed isopentane bath. Tissue blocks were stored at -70°C until 5- to 7-μm sections were prepared and mounted on gelatin-coated glass microscope slides. After acetone fixation, sections were stained with dilutions of heat-inactivated rat serum, washed and incubated with FITC conjugated goat anti-rat IgM (Accurate, Westbury, NY) or sheep anti-rat IgG (Serotec, Oxford, England). Sections were then examined using a Zeiss D-7982 Oberkochen microscope (Carl Zeiss Inc., Thornwood, NY).

Experimental Design

Lewis rats were divided into three groups of corneal graft recipients. One group had no manipulation before corneal transplantation (naive recipients). The other two groups received orthotopic skin grafts from Hartley guinea pigs (xenosensitized recipients) or from ACI rats (allosensitized recipients). Ten to 12 days after skin grafting, recipient rats received an orthotopic corneal graft from Lewis rats (isografts), ACI rats (allografts), or Hartley guinea pigs (xenografts). Two grafts from each donor category were performed on the same day. Graft survival was analyzed by a Mantel-Haenszel program, and differences between the groups were analyzed by Student’s t-test.

RESULTS

Corneal Grafts in Naive Recipients

The results of corneal grafts into naive recipients are shown in Figure 1. All 8 isografts survived throughout the 30-day observation period. Five of 6 ACI allografts were rejected in this high-responder strain combination, with a mean survival time of 11 days. All 10 Hartley guinea pig xenografts were rejected between 6 and 9 days, with an average survival time of 8 days. Though the survival of xenografts was significantly shorter than that of both isografts (P < 0.001) and allografts (P = 0.016), it was strikingly longer than the brief survival time of heart grafts in the same species combination, which survived 17 ± 4 min. The histologic features of the rejected corneal grafts included a massive diffuse infiltration of mononuclear cells and polymorphonuclear cells, accompanied by severe edema throughout the stroma, destruction of the endothelial layer, and epithelial sloughing (data not shown). An extensive analysis of the histopathologic characteristics of rejecting corneal xenografts and the phenotypic composition of the infiltrating cells is in progress.

Corneal Grafts in Allosensitized Recipients

To determine if presensitization to allogeneic antigens would lead to cross-reactivity with xenogeneic antigens expressed on the cornea or heightened immune
responsiveness in recipients, a group of Lewis recipients was preimmunized with fully allogeneic ACI skin grafts before receiving corneal transplants. As shown in Figure 2, allosensitization had no effect on the survival of Lewis isografts \((P > .05)\). Xenogeneic corneal grafts were also unaffected by allosensitization \((P > .05)\). However, rats that received ACI skin grafts exhibited a trend of decrease in the mean survival time of subsequent ACI corneal allografts from 11 to 8 days \((P = .06)\).

Corneal Grafts in Xenosensitized Recipients

To determine if the prolonged survival of guinea pig corneal grafts was simply the result of the isolation of the avascular graft site from the immune system, a group of Lewis recipients was preimmunized with Hartley guinea pig skin grafts. These recipients had high levels of circulating guinea pig reactive IgM and IgG as measured by fluorescence cytometry at the time they received a corneal graft (see below). Both corneal isograft and allograft survival were unaffected by xenosensitization \((P > .05)\) compared to naive recipients (Fig. 3). However, xenogeneic corneal grafts were rejected significantly more quickly \((P < .001)\) in the xenosensitized recipients (5 days) than in the naive recipients (8 days), with signs of rejection occurring as early as the third postoperative day including mild-to-moderate graft edema and opacification with significant involvement of the recipient cornea as well (Fig. 4a). By contrast, allografts in xenosensitized recipients at day 3 were clear, with normal levels of trauma-associated graft opacification at the wound margins and with the recipient iris and pupil easily seen through the graft (Fig. 4b). A summary of survival in all 9 donor-recipient combinations is shown in Figure 5.

Xenoantibody Production

Serum samples from corneal graft recipients were heat inactivated and used to stain guinea pig lymph node cell targets as a standard assay used to measure NAb.22 At time 0 before grafting, recipients had only low levels of IgM and undetectable IgG NAb (Fig. 6). However, after rejection of the xenografts but not the allografts, the levels of both guinea pig reactive IgM and IgG antibodies were much higher (Fig. 6). Similar results were obtained when guinea pig platelets or erythrocytes were used as targets (data not shown). Figure 7 demonstrates that in the recipients presensitized by guinea pig skin grafts, high levels of IgM and IgG were present in the circulation at the time of corneal transplantation.

Expression of Xenoantigens on Cornea

To determine if the prolonged survival in naive recipients of xenogeneic corneal grafts, relative to cardiac grafts, was the result of a paucity of xenoantigens on the donor cornea, sections of normal guinea pig cornea were stained with serum from corneal graft recipients. Lewis rats that had rejected xenogeneic corneal grafts contained serum IgG and IgM, which reacted strongly \((4+)\) with guinea pig cornea (Figs. 8a and 8b). Epithelial cells exhibited moderate diffuse staining with a superimposed brighter, granular staining pattern. Stromal staining was also present and was confined to cellular elements. No staining of endothelium or of the endothelial or epithelial basement membranes was detected. Serum from Lewis rats that rejected allogeneic corneal grafts displayed weak but significant staining \((1+)\), with a distribution similar to
A FIGURE 4. Clinical appearance of corneal grafts in xenosensitized recipients. (a) On the third postoperative day, a xenograft is already completely opaque with moderate-to-severe edema. (b) An allograft at the same time is still completely clear with an easily detectable pupil.

that seen for xenogeneic corneal recipients. However, an identical pattern and intensity of staining was obtained when serum from naive rats was used (Fig. 8c), indicating that reactivity was against antigens recognized by natural antibodies.

When sections of guinea pig heart were stained with serum from recipients of allogeneic and xenogeneic corneal grafts, a vascular staining pattern was observed (Fig. 9). Again, sections stained with the serum of a xenograft recipient were more intense than, but showed the same pattern as, sections stained with serum from allogeneic graft recipients or naive rats.

DISCUSSION

Vascularized discordant xenogeneic transplants are rejected hyperacutely within minutes to hours. By contrast, we found that orthotopic corneal grafts in the well-characterized guinea pig-to-Lewis rat species combination survived for up to 8 days before being

SURVIVAL OF CORNEAL TRANSPLANTS

FIGURE 5. Summary of guinea pig xenograft survival in naive, xenosenisitized, and allosensitized Lewis rats.

XENOREACTIVITY OF SERUM FROM NAIVE CORNEAL GRAFT RECIPIENTS

FIGURE 6. Xenoreactivity of serum from naive corneal graft recipients. Guinea pig lymph node cells were stained with serial dilutions of serum from corneal graft recipients and analyzed by flow cytometry.
not express the same or as many xenogeneic antigens. However, inflammation after corneal transplantation can lead to upregulation of class II major histocompatibility complex antigens on corneal tissues, and perhaps the same is true of xenoantigens. Thus, a grafted guinea pig corneal endothelium may express more targets for NAb binding than a normal one.

Corneal grafts are exposed to immunoglobulins through both the aqueous humor and tears; the potential impact of this exposure may be lessened by limited quantities of antibody compared with vascularized grafts and limited exposure to cellular or humoral cofactors such as C' components. The importance of alternative C' pathway activation in this discordant model relative to direct activation by NAb may also account for the prolonged survival of guinea pig corneal xenografts by rats.

The prolonged survival of xenogeneic corneal grafts holds promise for their future clinical applica-

rejected by naive recipients even in the absence of any immunosuppression. Immunization of the recipients with allogeneic skin grafts did not affect the survival of subsequent xenogeneic corneal grafts. However, preimmunization with xenogeneic skin grafts stimulated xenobinding responses and significantly shortened survival times of xenogeneic corneal grafts from 8 days to 5 days. This indicates that the prolonged survival of xenogeneic corneal grafts is not merely the result of isolation from the recipient immune system and that xenografts can be rejected in an accelerated manner, faster than that previously reported for allografts in any experimental model.

The lack of hyperacute rejection may be attributed to the paucity of cornea-reactive NAb, or target antigens, to sequestration of the graft from NAb exposure, or to the absence of other proteins or cells that act as cofactors with NAb. Immunofluorescence studies demonstrated that the serum of naive rats and rats that received an allograft contained both IgM and IgG, which bound to guinea pig cornea, indicating that xenogeneic antigens recognized by NAb are expressed on the guinea pig cornea. The low level of staining suggests that the xenogeneic antigens or NAb are present at low levels. By contrast, serum from rats that rejected guinea pig corneal grafts stained normal guinea pig cornea much more strongly. The brightest staining was observed on epithelial and stromal cells and represented genuine antibody binding rather than Fc receptor-mediated binding because the level of binding was much higher than that obtained with naive or alloimmune sera. No endothelial staining was present, which suggests this critical layer of the cornea may

FIGURE 7. Xenoreactivity of serum from xenoimmunized recipients. Lewis rats received guinea pig skin grafts 10 days before corneal transplantation. Guinea pig lymph node cells were analyzed as in Figure 6.

FIGURE 8. Expression of xenoantigens on the cornea. (a) Sections of normal guinea pig cornea were incubated with serum from the recipient of a xenograft and stained with goat anti-rat IgG. Cellular elements in the epithelium and stroma stained positively (original magnification, 250x). (b) A higher magnification of the epithelium is shown in this panel (original magnification, 400x). (c) Epithelium of a cornea incubated with serum from the recipient of an allograft and stained with goat anti-rat IgG (original magnification, 400x). Only faint staining is detectable.
FIGURE 9. Expression of xenoantigens on the heart. Sections of normal guinea pig heart were stained as in Figure 7, with (a) serum from a rat that had rejected a corneal xenograft (original magnification, 400x) or (b) an allograft (original magnification, 100x).

The 8-day survival time of these grafts is not inconsistent with cellular rejection, for which well-established therapies already exist. The finding that presensitization to allogeneic antigens did not lead to accelerated rejection of subsequent xenografts is also encouraging. Allosensitization, especially from prior graft rejection, has been reported to be associated with higher risk for graft rejection of vascularized organs and corneal grafts. Xenografts, therefore, may represent a viable option for those patients who have already rejected a corneal allograft or who have been sensitized by pregnancy or transfusion.

In summary, we have examined corneal graft rejection in a well-characterized discordant rodent species combination where hyperacute rejection of vascularized organs occurs. Our findings indicate that the cornea expresses xenoantigens, that rejection of corneal xenografts promotes xenoantibody production, and that presensitization to skin xenografts elevates xenoantibody levels and significantly accelerates subsequent corneal xenograft rejection. Unlike vascularized allografts, however, corneal xenografts are not hyperacutely rejected, even in presensitized recipients. These findings provide the basis for further studies of the feasibility of discordant cornea xenotransplantation.

Key Words

corneal transplantation, xenotransplantation, hyperacute rejection, antibody response

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References