Fate of Lyophilized Xenogeneic Corneal Lenticules in Intrastromal Implantation and Epikeratophakia

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The antigenicity of intrastromal and epikeratophakia xenografts of lyophilized corneal tissue was evaluated in nonimmune and immune recipients. Lyophilized feline lenticules were implanted into intrastromal pockets in unsensitized rabbits and rabbits sensitized to the donor cat. In both cases, the grafts remained clear. Sensitized rabbits with clear intrastromal grafts received fresh tissue penetrating keratoplasty grafts from the same donor cat, placed adjacent to the intrastromal grafts. The fresh tissue penetrating keratoplasty grafts were rapidly rejected, while the lyophilized intrastromal grafts remained clear. Cats sensitized to rabbits received lyophilized and rehydrated epikeratophakia grafts shaped from rabbit cornea; these lyophilized grafts also remained clear for the 3-month period of the study. The results indicate that lyophilized and rehydrated corneal stroma, which is devoid of living cells, is not antigenic and is not subjected to immunologic attack, even in cases where the donor and host are of different species and the host has been previously immunized to the donor. Invest Ophthalmol Vis Sci 28:555–559, 1987

The development of surgical procedures for the correction of refractive errors and corneal abnormalities is an area of considerable interest and clinical significance. The techniques of epikeratophakia and keratomileusis have proved to be sight-restorative and sight-saving for many individuals. Unfortunately, the widespread adoption of these techniques outside the United States may be limited because of the limited availability of human-derived corneal tissue. There are numerous studies in which various synthetic intrastromal corneal implants have been used to correct refractive errors. These implants still appear to have major limitations and none are routinely used in humans. Thus, the preferential use of lenticules of human cornea will continue to be warranted. However, even in such an ideal circumstance, ie, human donor tissue in the human eye, the possibility of a host response to a lenticule from an allogeneic donor has not been adequately ruled out.

In the current study, the intent was to establish the “worst-case” scenario in which non-immune or immune members of one species were given implants from a widely divergent species and the fate of the xenografts observed. No incompatibilities indicative of an immune response were observed in response to lyophilized tissue grafts, even when the recipient had been previously sensitized to the donor from which the graft was obtained.

Materials and Methods

Animals

Animals involved in this investigation were cared for and used according to the ARVO Resolution on the Use of Animals in Research. New Zealand white rabbits, weighing 2–3 kg, and young adult cats of both sexes were used. All surgical procedures were conducted on fully anesthetized animals. Rabbits were given intramuscular injections of ketamine hydrochloride (30 mg/kg body weight) and xylazine hydrochloride (3 mg/kg body weight). Cats were given intramuscular injections of ketamine hydrochloride (30 mg/kg body weight), chlorpromazine (0.5 mg/kg body weight), and atropine (0.1 mg/kg body weight). A retrobulbar injection of 1 cc of 2% lidocaine hydrochloride was given to cats to provide proptosis. Corneal anesthesia was maintained with 0.5% proparacaine hydrochloride drops in both rabbits and cats.

Immunologic Sensitization

Sensitization was accomplished by intravenous immunization with leukocytes. Immunity of cats to rabbit (and rabbit to cat) histocompatibility antigens was determined by a serum cytotoxicity assay which is uni-
versally used in clinical organ transplant settings to identify pre-sensitized patients.

Pairs (one each) of rabbits and cats were bled by cardiac puncture into heparinized 12 ml syringes. Anticoagulated blood (10 ml) was transferred to sterile conical 15 ml centrifuge tubes and centrifuged at 900 × g for 10 min. The buffy coat was collected by gentle aspiration into a sterile Pasteur pipette and transferred to a second sterile centrifuge tube. The buffy cells were washed twice in sterile Hanks balanced salt solution (HBSS) (GIBCO, Grand Island, NY) and resuspended in 5 ml of HBSS for counting. Total nucleated cell counts were performed in a hemacytometer counting chamber. The average nucleated cell yield was 43 × 10^6 from 10 ml of rabbit blood, and 59 × 10^6 from 10 ml of cat blood. Each pair of animals was reciprocally immunized three times at weekly intervals. Rabbits received 40 × 10^6 cat leukocytes in 1 ml HBSS in the marginal ear vein. Cats received 40 × 10^6 rabbit cells in 1 ml HBSS by the intracardiac route. Serum samples for cytotoxic antibody testing were obtained before immunization and seven days after the last immunization.

The immune status of leukocyte-injected rabbits and cats was determined by means of a cytotoxicity assay. Leukocyte donors were bled for 5 ml of heparinized blood. Lymphocytes were purified using a Ficoll-Hypaque separation technique. The blood was diluted 1:1 in HBSS and 5 ml volumes layered onto 5 ml of Ficoll-Hypaque (Sigma, St. Louis, MO), density 1.077, in 15 ml centrifuge tubes. The tubes were centrifuged at 400 × g for 30 min and the leukocyte band at the interface was collected, washed, and counted. Pre- and postimmunization serum samples were subjected to 12 serial twofold dilutions in microtiter tray wells. Twenty-five μl volumes of the appropriate purified leukocyte suspension were added to each well, along with 25 microliters of a 1:10 dilution of guinea pig complement. The microtiter plates were incubated for 45 min at 30°C, and the percent of dead cells in each well determined using the vital dye, trypan blue.

Tissue Preparation

**Intrastromal grafts:** Sterile technique was used. With the animals under anesthesia, a partial thickness, stromal button was removed from the left corneas of cats. An 8.0 mm trephine set at 0.25 mm was used. The epithelium was removed with a large stainless steel blade (No. 15 Bard Parker, American Scientific Products, Harahan, LA) prior to trephination. The resulting corneal defect was filled with a lyophilized, lamellar graft from a donor cat that was not included in this study. The corneal buttons were placed in McCarey-Kaufman medium and stored at 4°C for no more than 2 days, then frozen and lathed using a Barraquer cryolathe (Steinway Instruments, San Diego, CA). No intermediate solutions containing glycerine, dyes, or dimethylsulfoxide were used in this study. The dimensions of the lenticules were: 6.0 mm diameter, 0.2 mm (plano) thickness. The lenticules were lyophilized and stored in vacuum-sealed vials at room temperature for no more than 1 week. The lathing, lyophilization, and storage of the intrastromal lenticules was done according to the processing currently used for epikeratophakia in humans.

**Epikeratophakia grafts:** With sterile technique, the epithelium was removed from the corneas of anesthetized rabbits. A 10 mm trephine was used to excise a full thickness corneal button. The animal was then sacrificed. The epithelium and endothelium were removed with a cotton-tipped swab. The button was stored in McCarey-Kaufman medium for no more than 2 days, then frozen and lathed on the Barraquer cryolathe. The dimensions of the lenticules were: 9.5 mm diameter, 0.2 mm (plano) thickness. The epikeratophakia lenticules were lyophilized and stored at room temperature in vacuum-sealed vials for no more than 1 week.

**Tissue for penetrating keratoplasty:** With sterile technique and the animals under anesthesia, the right eyes of cats who had previously served as donors of intrastromal grafts underwent a full thickness trephination of a 6 mm button. The animals were then sacrificed. The fresh full thickness button was transplanted immediately into the corresponding host rabbit cornea.

**Surgery**

**Intrastromal grafts:** Corneal stromal pockets were created centrally in recipient rabbits using a fine-tipped stainless steel blade (No. 64 Beaver, Storz, St. Louis, MO). The lenticule was rehydrated with sterile balanced salt solution and gentamicin (200 micrograms/ml) and inserted into the stromal pocket. Nylon sutures (10-0) were used to close the wound. These were removed 1–2 weeks after surgery. Rabbits were examined with a slit lamp three times a week for 2 weeks, then weekly for a period of 3 months. Photographs were taken throughout the study. In animals undergoing sensitization, surgery was performed within 1 week of the most recent sensitization.

**Penetrating keratoplasty:** A full thickness corneal button was excised with a 6.0 mm trephine from the same rabbit cornea that had previously received an intrastromal graft. This resulting recipient bed was located between the limbus and the intrastromal graft. The anterior chamber was irrigated with heparin to prevent fibrin clot formation. A 6.0 mm fresh, full thickness feline corneal button was sutured into this bed with interrupted 10-0 nylon sutures. Gentamicin (2 mg) was injected in the subconjunctival space. One
drop of 5% homatropine and neomycin ointment were placed in the eye at the end of surgery. The sutures were removed 2–3 weeks after surgery. The eyes were examined with a slit lamp every 2 days for 3 weeks, then twice a week for 4 weeks.

*Epikeratophakia grafts:* The epithelium was removed from the host cornea with a large stainless steel blade. A 7.5 mm trephine set at 0.25 mm was used to create an annular incision. A 0.5 mm keratectomy was created just inside the incision. The epikeratophakia graft was sutured in place with 16 interrupted 10-0 nylon sutures. Gentamicin (2 mg) was injected in the subconjunctival space. One drop of 5% homatropine and neomycin ointment were placed in the eye. The lids were sutured shut with 4-0 silk sutures. The tarsorrhaphy was removed 3 days after surgery. The epithelium was found to be intact. Slit lamp examinations were done three times a week for 2 weeks, then once a week for 2 months.

**Histology**

At the end of the observation period, recipient animals were sacrificed. The corneas were excised at the limbus and immediately fixed in formalin. The fixed tissue was embedded in plastic, sectioned, and stained with hematoxylin and eosin and periodic acid-Schiff. Sections were examined under light microscopy for the presence of a host cellular reaction to the intrastromal, penetrating keratoplasty, and epikeratophakia grafts.

**Results**

**Lyophilized Intrastromal Xenografts in Nonimmune Hosts**

Six lyophilized and rehydrated stromal implants were placed in the corneas of nonimmune rabbits (Table 1). Four remained clear and unremarkable during the 3-month observation period. Two grafts became infected and were removed. Histologic examination of sections of stromal implants revealed the benign nature of the implant site. Cat stromal tissue was observed to be in direct contact with that of the rabbit. A few keratocytes were present within the implant.

**Lyophilized Intrastromal Xenografts in Immune Hosts**

Five rabbits were sensitized to cats by the protocol outlined above. Sensitization was achieved by three injections of blood leukocytes. The results of the cytotoxicity assay proved that the leukocyte injections established a significant level of immunity in the rabbits (Fig. 1). Serum samples obtained before immunization gave no evidence of immunity.

Lyophilized and rehydrated stromal implants were implanted into immune hosts within 1 week of establishing their state of sensitization. All five of these implants remained clear and failed to elicit a host response. These grafts did not look significantly different from those placed on the corneas of nonimmune animals throughout the entire 2-month observation period. Histologically, these xenogeneic implants gave no evidence of being subjected to an inflammatory or immune reaction.

**Xenogeneic Penetrating Keratoplasties in Immune and Nonimmune Hosts**

Six rabbits, four immune and two nonimmune, which had received intrastromal implants, were given fresh tissue penetrating keratoplasty grafts obtained from the same donor cats (Table 2). Four of these penetrating keratoplasty grafts (two in immune recipients and two nonimmune recipients) became markedly vascularized and were rejected (Fig. 2). Of these, two grafts were actually extruded, while two others were gradually absorbed and replaced by fibrous scar tissue. The rejection process did not involve the adjacent in-
Table 2. Adjacent lyophilized intrastromal and fresh feline penetrating grafts into rabbit cornea

<table>
<thead>
<tr>
<th>Status of graft</th>
<th>Clear graft</th>
<th>Rejected graft</th>
<th>Infected graft</th>
<th>Wound dehiscence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh penetrating graft (N = 6)</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lyophilized intrastromal graft (N = 6)</td>
<td>6</td>
<td>0</td>
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intrastromal implants; these remained clear for the 2-month observation period. One graft became completely dehisced 4 days after surgery. Another graft became infected 1 month after surgery and was removed.

Histologic examination of sections of penetrating keratoplasty grafts in corneas removed at 2 months showed a zonal granulomatous reaction completely surrounding the tissue (Fig. 3). Some of the penetrating keratoplasty grafts were shrunken and had retrocorneal and subepithelial fibrous tissue, while others were extruding. Cell types present included plasma cells, lymphocytes, macrophages, and giant cells. There were scattered chronic inflammatory cells within the stroma of the graft; however, the host reaction was most marked at the edges of the graft where the collagen was being destroyed and replaced by fibrous tissue. There was no evidence of an inflammatory reaction in the adjacent intrastromal lyophilized implant.

Fig. 2. Rejected opaque penetrating keratoplasty fresh tissue graft (below), with adjacent clear lyophilized intrastromal graft (center).

Fig. 3. Extruding penetrating keratoplasty fresh tissue graft (below) adjacent to lyophilized intrastromal graft (above). Note inflammatory cells surrounding fresh tissue but not lyophilized tissue (Hematoxylin and eosin, ×40).

Epikeratophakia Xenogeneic Grafts in Immune Hosts

Three cats received epikeratophakia grafts from rabbits to which they had been sensitized according to the protocol described above. All grafts remained epithelialized after the tarsorrhaphy was removed. One graft became infected and vascularized during the 2-month observation period. Two grafts remained clear throughout the 3-month period of the study.

Discussion

The purpose of this study was to determine whether lyophilized corneal tissue is significantly antigenic. In human epikeratophakia, donor corneal tissue is frozen and lyophilized and consists of killed keratocytes1 within a stromal collagen matrix. The donor tissue is devoid of epithelium and endothelium.

When such tissue was implanted into a central corneal pocket in both nonimmune and immune xenogeneic hosts, the implants remained clear (See Table 3 for summary of results). Willey et al demonstrated antibody production in host rabbits after fresh and lyophilized lenticules were implanted at the limbus but not centrally.2 We demonstrated specific immunity to donor antigens in host rabbits prior to implantation in order to establish the afferent arm of an immune response, the “worst-case” scenario for the implantation of a lenticule. These lenticules remained clear.

We worsened the situation by placing a fresh, full-
thickness penetrating keratoplasty graft, whose cells bore the original donor antigen, adjacent to the intrastromal lenticules. We hoped to establish the effector arm of an immune response to see if the graft reaction we induced would involve the lyophilized lenticule as well as the fresh-penetrating keratoplasty graft. The fresh graft was rejected in a well-documented manner, including the development of florid vascularization, while the lyophilized lenticule remained clear.

It is thought that freezing corneal tissue reduces its antigenicity by killing cells bearing transplantation antigens.3,4 Graft reactions to tissue prepared in this manner, as shown by Lorenzetti and Kaufman,5 may be due to inert stromal elements and not living cells. Bovine collagen dermal implants in humans are relatively inert, yet are still capable of producing adverse reactions and antibody production.6 Glutaraldehyde-treated bovine heart valves are capable of stimulating cellular immunity when placed subcutaneously in rabbits.7

During lyophilization, tissue is frozen. Water is removed from the tissue in the frozen state by a strong vacuum. This does alter the structural components8 and apparently renders the tissue less antigenic than fresh tissue.9 Cryopreservation, in which cells are maintained in a viable state after freezing, does not appear to reduce antigenicity.4

Lyophilized epikeratophakia lenticules processed and grafted as in human surgery were placed onto corneas of immunized hosts to establish a worst case situation. These lenticules epithelialized rapidly and remained clear. The lyophilized intrastromal corneal lenticules were also well-tolerated, and remained clear. The failure of these xenogeneic grafts composed of lyophilized tissue to elicit either clinical or histopathologic reactions establishes the lack of immunogenicity of lyophilized corneal tissue. The absence of antigenic stimulation is essential for the survival of such grafts used in humans for refractive correction, as in epikeratophakia. Furthermore, the lack of an immune response to the lyophilized tissue implies that persons receiving such tissue are not exposed to an enhanced risk of graft rejection should they require penetrating keratoplasty in the future.

Key words: cornea, rabbit, cat, immune reaction, lyophilized stroma, epikeratophakia

References