An Improved Method of Intralamellar Keratoplasty in Rats

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Intralamellar keratoplasty in inbred rats is a valuable method for the investigation of corneal allograft rejection. Present methods of intralamellar corneal grafting are tedious and time-consuming. In addition, induction of neovascularization, a prerequisite to inducing graft rejection, is variable. In an effort to overcome these problems, the authors have developed an improved method of intralamellar keratoplasty. Intralamellar pockets are formed by introducing a 30-g needle into the corneal stroma near the limbus. Approximately 10–25 μl of lipopolysaccharide (LPS) (100 μg/ml) is injected into the stroma, forming a stromal bleb. This bleb is incised to form a pocket, resulting in the leakage of the injected liquid. The intralamellar pocket formation is completed by dissecting any remaining stromal fibers in the area of former bleb. Once the pocket has been formed, grafts of corneal tissue are inserted, and the incision is closed. This method of keratoplasty has the following advantages over previously reported methods: (1) It is more rapid and less tedious because the formation of the corneal bleb protects the anterior chamber from being invaded during the corneal incision; (2) It leads to a reproducible induction of neovascularization in every cornea so treated; (3) It results in a higher frequency of allograft rejection.

Materials and Methods

Animals

Female rats (150–200 gm) of the inbred Lewis (RT 1-1) and Wistar-Furth (RT 1-2) strain were used. The Lewis strain was used as a common recipient and always received isografts in one cornea as a control and allografts (Wistar) in the other cornea. Our utilization of animals in this investigation adhered to the ARVO Resolution on the Use of Animals in Research.
Chloramphenicol eye drops were applied daily for the first 4 days. One 9-0 silk suture that was removed at 48 hr. required. The remaining stromal fibers in the bleb were entered, and no stabilizing or proptosing sutures were required. The remaining stromal fibers in the bleb were dissected with a #66 beaver blade (Fig. 1D), completing the formation of the intrastromal pocket (Fig. 1E). One half of a 2-mm full-thickness corneal button was then placed in the pocket (Fig. 1F) and the pocket closed with one 9-0 silk suture that was removed at 48 hr. Chloramphenicol eye drops were applied daily for the first 4 days.

**Corneal Grafting**

Full-thickness corneal donor tissue was obtained with a 2-mm trephine after killing the rats with an overdose of sodium pentobarbital. The 2-mm, full-thickness corneal button obtained was placed in sterile, normal saline and divided into two equal pieces. Recipients were anesthetized by injecting pentobarbital (20 mg/kg body wt) intramuscularly. They received an injection of ketamine hydrochloride (20 mg/kg body wt) intramuscularly 10–15 min later. After surgical anesthesia was obtained, one drop of proparacaine hydrochloride (0.5%) was placed in the recipient’s eye. The sclera was lightly grasped with a pair of tissue forceps (Fig. 1A), and 10–25 μl of either sterile LPS (100 μg/ml) (Sigma Chemical Co.; St. Louis, MO) or sterile saline was injected into the stroma of the cornea using a 30-g needle bent to follow the acute curvature of the rat cornea. The injection of fluid results in the formation of a stromal bleb (Fig. 1B). The technique of injection is very much like the technique used to make an intradermal injection, and the bleb that is formed is equivalent to the wheal that is formed with an intradermal injection. The corneal incision was made directly over the bleb (Fig. 1C), resulting in the leakage of the fluid within the bleb. When an incision was made in this manner, the bleb resulted in a large margin of safety so that the anterior chamber was virtually never entered, and no stabilizing or proptosing sutures were required. The remaining stromal fibers in the bleb were dissected with a #66 beaver blade (Fig. 1D), completing the formation of the intrastromal pocket (Fig. 1E). One half of a 2-mm full-thickness corneal button was then placed in the pocket (Fig. 1F) and the pocket closed with one 9-0 silk suture that was removed at 48 hr. Chloramphenicol eye drops were applied daily for the first 4 days.

**Induction of Vascularization and Assessment of Allograft Rejection**

Recipient eyes that received intrastromal injections of normal saline (10–25 μl) also received subconjunctival injections (50 μl) of LPS (100 μg/ml) immediately following surgery to induce corneal vascularization. Recipient eyes receiving intrastromal injection of LPS required no further stimulus to induce neovascularization. Transplant success was evaluated by grading the graft under a stereo microscope at 2–3-day intervals. Corneal clarity was graded 0–3, with 0 indicating a clear cornea and 3 indicating a completely opaque cornea. After dilating the pupil with 1% atropine, vascularity was graded 0–4, with 0 indicating no vessels in the cornea, 1 indicating vessels entering the cornea from the limbus but not into the incision, 2 indicating vessels into the area of the incision but not into the graft, 3 indicating vascularization of the graft, and 4 indicating massive vascularization of the graft from 360 deg. This grading system is a slight modification of the system used by Lang et al. Grafts were scored in a single blind manner and histologic sections were routinely made to confirm stereomicroscopic evaluation of rejection. Grafts with histologic evidence of rejection always had total scores (vascularity + clarity) greater than 3. Rejection was defined as opacification of a previously clear graft, disruption of the normal cellular architecture of the graft, infiltration of the graft by lymphocytes, and the presence of blood vessels in the graft.

**Experimental Design**

To test this procedure, two groups of rats encompassing four experimental designs were established. In one group of 14 animals, the intrastromal pockets were formed by the injection of LPS. These animals then received an isograft in one cornea (group A1) and an allograft in the other (group A2). The pocket in the second group of seven rats was formed by an intrastromal injection of sterile saline. These animals received an isograft in one cornea (group B1) and an allograft in the other (group B2). Vascularization in these corneas was subsequently induced by subconjunctival injection of LPS.

**Results**

When isografts are placed in intralamellar pockets formed by intrastromal injection of LPS (group A1) or intrastromal injection of normal saline with subconjunctival LPS injection (B1), there is an initial rise in total score (vascularity + clarity), which peaks by day 9 and reaches maximal total mean scores of 5.2 and 4.2 for groups A1 and B1 respectively (Fig. 2). The total mean scores then begin to drop off and reach levels below 3 by day 20. Histologic criteria for graft rejection were not met by any of the isografts, regardless of the method used to form the intralamellar pocket, and the differences between the total mean scores and mean vascular scores for the groups A1 and B1 were not statistically significant at any time.

Allografts placed into intralamellar pockets formed by intrastromal injection of LPS (A2) or normal saline injection with conjunctival injection of LPS (B2) both show an initial rise in total mean scores that again peaks by day 9 (Fig. 3). Allografts in group A2 reached a peak total mean score of 6, whereas allografts of group B2 had a peak total mean score of 4.9. As was seen with the isograft groups, the total mean scores of the allografts begin falling off after peaking. However, the total
Fig. 1. Steps involved in preparing intralemellar pocket for grafting corneal tissue: (A) 30-gauge needle introduced into the corneal stroma; (B) 10–25 µl fluid introduced into the cornea to form a bleb; (C) the bleb is incised with a diamond knife to form a pocket, which is further dissected; (D) histological sections of such a pocket; (E) demonstration of the incision made by the knife and the defect in the corneal stroma; (F) a graft is prepared for insertion into the pocket following placement of a single suture.
mean scores in group A2 remained high and eventually plateaued at a total mean score of nearly 5, which correlated with 100% rejection. Allografts in group B2 had total mean scores that eventually plateaued at about 2, which corresponded with a rejection frequency of 65%. Previous studies in our lab utilizing the method of Lang et al for intralamellar keratoplasty resulted in a 70% frequency of graft rejection and a virtually identical total mean score versus date-response curve. When one examines Figure 4, depicting the mean scores of vascularity with time for both allograft groups (A2 and B2), it can be seen that the intrastromal injection of LPS results in a more prolonged and more pronounced neovascularization of the allograft. When the two-tailed t-test for statistical analysis was utilized, the difference in the mean vascular scores between groups A2 and B2 were shown to be statistically significant (P < 0.02) on day 17, whereas the difference in the total mean scores became significant on day 29.

Figures 5 and 6 are representative photomicrographs of hematoxylin and eosin (H & E) stained sections of corneas from groups A1 (isograft) and A2 (allograft) respectively. The isograft in Figure 5 shows no evidence of rejection (note the Descemet's membrane of graft). The allograft in Figure 6 contains blood vessels and lymphocytes and has architectural disruption of the normal stromal intralamellar pattern indicating rejection. No graft epithelium or endothelium could be identified in H & E stained histologic sections of cornea after 1 wk. However, the intralamellar grafts are easily identified microscopically by the increased width of the cornea in the graft area, the presence of a Descemet's membrane in some, but not all sections of each cornea, and frequently by a distinguishable circumferential graft-host tissue margin.

Throughout these experiments we observed that the intrastromal injection of LPS stimulated a reproducible vascularization of the cornea that covered an area equivalent to the area of the original corneal bleb regardless of whether the graft was an isograft or an allograft. The pattern of vascularization seen with sham-operated controls (corneas subjected to intrastromal LPS injection and pocket formation without graft placement) was similar to that observed in group A1 reaching maximum vascularity by day 10 and diminishing to barely perceptible levels by day 20. In general, the isografts became surrounded by blood vessels that did not invade the graft and became ghost vessels within 2–3 weeks. When allografts were present, however, the surrounding blood vessels invariably invaded the graft and remained patent.
Discussion

We have developed a rapid, simple method of performing intralamellar keratoplasty in inbred rats that results in consistent neovascularization and rejection of virtually 100% of corneal allografts. We believe the reason for this high frequency of allograft rejection is the consistent and prolonged neovascularization of allografts that are placed into intralamellar pockets formed by intrastromal injection of LPS. LPS is known to stimulate the neovascularization of corneas when it is injected intrastromally, and central corneal intrastromal injections of LPS have been shown to result in the rejection of nearly 100% of previously successful corneal allografts. Our method of intralamellar corneal grafting also utilizes intrastromal injections of LPS; however, the corneal incision results in leakage of the LPS out of the corneal bleb, and very little LPS would be expected to remain within the corneal stroma. The exact mechanism for the improved neovascularization of allografts utilizing our method is unclear; however, we have histologic evidence suggesting that even after the release of the LPS from the intrastromal bleb following the corneal incision, small pockets of LPS remain trapped within the stroma. We also postulate that the presence of allo-antigens present on the allografts are the stimulus responsible for the vascularization of the graft itself. Lymphocytes that are stimulated by the presence of alloantigens can produce lymphokines that have angiogenic properties, and may play a role in the prolonged vascularization observed in group A2. Recently, we have begun utilizing our technique of intrastromal bleb-formation to simplify the formation of a stromal flap as described by Mackintosh. This has resulted in greater ease of intact, full-thickness graft placement.

This improved procedure for keratoplasty in the rat should provide a method for an indepth study of the mechanisms of corneal graft rejection. This in turn might enhance our ability to manipulate this response and increase graft survival in those patients at risk for failure. Studies are underway in our laboratory to this end.

Key words: cornea, intralamellar keratoplasty, transplantation, graft rejection, inbred rats

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References