Histopathology of Rejected Orthotopic Corneal Grafts in the Rat

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We have used an orthotopic graft model in the rat to study the histologic characteristics of corneal allograft rejection. Unrejected allogeneic grafts could not be distinguished from clear syngeneic grafts. Although donor Langerhans cells are necessary for the development of delayed-type hypersensitivity (DTH), the histopathological characteristics of rejecting corneal allografts in immunologically naive hosts were identical regardless of the presence or absence of donor Langerhans cells. By contrast, preimmunization had a dramatic effect on the histology of graft rejection. Untreated allografts placed onto pre-immunized recipients underwent a marked cellular necrosis accompanied by minimal inflammation that easily distinguished these grafts from the previous groups. These results suggest that neither the presence nor absence of DTH responsiveness correlates with the histopathological events that accompany corneal graft rejection. However, preimmunization leads to a different histologic pattern of rejection that is characterized by an intense cellular necrosis. Invest Ophthalmol Vis Sci 30:413-424, 1989

Immunologic rejection remains the leading cause of graft failure in human corneal transplants. Several different species of animals have been employed to study this problem. Rabbits were used by Mau-menee2 and also by Khodadoust and Silverstein 3, 4 in the earliest studies of immune rejection. These studies provided valuable information on the characteristics of rejection of a true orthotopic graft. The drawback of using the rabbit was the lack of understanding of that animal’s immune system. Streilein et al5 devised a new method of studying this problem by grafting cornea heterotopically on a vascularized dermal bed in mice. This model has been used extensively by our lab6-8 and others9 to analyze the immunobiology of the cornea. Treseler, Foulks, and Sanfilippo10-13 have used a similar model of heterotopic grafts in inbred rats to further our understanding of corneal antigenicity. The use of inbred mouse and rat strains for such grafting experiments permits exquisite immunological analysis of corneal allografts. However, heterotopic corneal allograft models share a common disadvantage of being unable to evaluate the role of the avascular corneal graft bed. Coster and Williams14 developed a rat model of corneal transplantation that eliminates these obstacles and combines the attributes of both the rabbit and mouse models. We have used this model to study the characteristics of rejection of true orthotopic transplants15 and have demonstrated the development of antigen specific cytotoxic T lymphocytes (CTL) in host animals that have rejected fully allogeneic corneas. In this model it was also shown that the infiltration of donor corneas with donor-derived Langerhans cells led to almost 100% rejection of these corneas and was necessary to induce delayed-type hypersensitivity (DTH) responses in the host. Thus, this model possesses the attributes necessary to understand the process of immune rejection. In this report we employed the rat orthotopic corneal allograft model to characterize the histopathological events that accompany corneal graft rejection and to correlate these findings with the appearance of specific systemic immunological responses.

Materials and Methods

Rats

Female Lewis (LEW) and Wistar-Furth (WF) inbred rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). These two strains differ at the entire major histocompatibility complex (MHC)
and are not congenic. All animals used were more than 8 weeks of age. In all experiments, corneas from Wistar-Furth donors were grafted onto Lewis recipients. Animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research.

Grafting

The technique used for penetrating keratoplasty has been previously reported. This is a brief review of the procedure. Animals were anesthetized with a combination of 100 mg/kg of a 50 mg/ml solution of ketamine (Parke-Davis, Morris Plains, NJ) and 60 mg/kg of a 33 mg/ml solution of promazine (Wyeth, Philadelphia, PA), both injected intramuscularly. Two sutures of 4-0 nylon were used to retract the eyelids. A subconjunctival injection of 0.05 ml of a solution containing 1 mg/ml atropine and 1:1000 epinephrine was used to obtain maximal dilatation of the iris. A 3 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 and the central 3 mm area removed. The donor graft was then sewn in the 3 mm opening in the center of the recipient cornea. Twelve interrupted sutures of 11-0 nylon (Alcon Surgical, Fort Worth, TX) were used for closure. Prior to tying the last suture, a small air bubble was placed in the anterior chamber of the eye using a 30-gauge blunt-tip needle. This was done to elevate the cornea away from the iris until the anterior chamber was reformed by the production of aqueous humor. Eight of these 12 sutures were removed on day 7. A topical antibiotic (Dow Chemical, Indianapolis, IN) was layered over and filled in these incisions (corneal facets) but instead results in migration of Langerhans cells. Significant numbers of Langerhans cells can be seen in the central corneal regions as early as 4 days and remained for at least 10 days (data not shown). Of equal importance was the lack of neovascularization of the cornea induced by this procedure. The presence of Langerhans cells in mouse cornea has been confirmed histologically using monoclonal antibodies against la antigens and also by ATPase staining.

Repeate these studies using rat cornea. MRC OX6 antibody (Serotec, Indianapolis, IN) was employed in the immunofluorescence staining and the technique of Bergstresser and Juarez was used for ATPase staining. Results obtained using rat corneas were identical to those seen in mouse cornea and previously reported. Untreated central corneas show only rare la Langerhans cells, but latex-treated corneas have a marked increase in the number of Langerhans cells present. In addition, no neutrophils, lymphocytes or macrophages were observed on these central areas. These latex-treated donor corneas were grafted onto recipient rats in the usual manner described above.

Histology

The grafted eyes were removed following euthanasia of the rat and were fixed overnight in Carson's formalin. Fixed eyes were progressively dehydrated to 95% alcohol and were embedded in glycol methacrylate (Sorvall, Wilmington, DE). Three micron sections were cut and were stained with hematoxylin and eosin (H&E) and periodic-acid-Schiff (PAS). Separate grafts of each type were examined histologically within 24 hr of clinical rejection, 48-72 hr after rejection, at 10 days post-rejection, and in other animals, several weeks after rejection was noted.

Clinical Observation

Animals were observed with a slit-lamp microscope at least twice weekly for signs of rejection. Opacity of the graft, edema of the graft and neovascularization of the recipient and donor cornea were scored as minimal, moderate or severe. If all three parameters became moderate or severe, the graft was recorded as rejected on that day. If the anterior chamber was not adequately reformed by 24 hr post-operatively, the animal was sacrificed and not included in the study. Any animal developing a mature cataract was also eliminated from the study as this made graft evaluation more difficult.

Results

Grafting Outcome

Of the 87 grafts reported here, only seven (8%) of the animals were eliminated from follow-up due to technical problems, such as infection, mature cata-
Fig. 1. Light micrographs of syngeneic Lew cornea grafted onto Lew recipient (day 10). Wound is well-approximated, nonvascularized, with minimal stromal infiltration of lymphocytes and neutrophils (lower left). Central graft is healthy (lower right) (upper H&E, X33; lower left H&E, X225; lower right H&E, X360).

Syngeneic grafts: Fourteen Lewis rats receiving syngeneic Lewis corneas were used as surgical controls. Following surgery these grafts had minimal edema which cleared by day 7. The recipient cornea was noted to become slightly vascularized between day 3 and day 7. Following removal of the last sutures on day 7, these grafts cleared completely and the vessels in the recipient cornea regressed. These grafts remained clear for as far out as 120 days post-surgery. When examined histologically at day 10, these grafts showed a minimal stromal inflammatory cell infiltrate consisting of neutrophils and lymphocytes and the anterior chamber was quiet, as shown in Figure 1. Even at this early stage the donor endothelium and epithelium were healthy and free of infiltrate and the wound margin was relatively quiet, as seen in the higher magnification photos of Figure 1. The lens shows minimal focal damage.

Allogeneic grafts: When fully allogeneic WF corneas were placed on Lewis recipients, 18 of 40 or 45% of these grafts remained completely clear as far out as 50 days postoperatively (Fig. 2). No rejections were observed after 46 days. These nonrejected grafts followed a course similar to the syngeneic grafts. They also had transient edema between days 3 and 7 and then proceeded to clear. At no point did these grafts appear rejected by the criteria stated in the Methods and Materials section. When examined as early as 2 weeks post-transplant these corneas were free of cellular infiltrate and the donor endothelium was free of any damage. Figure 3 shows the histology of these nonrejected allografts at 60 days postoperative. The graft was of normal thickness, the donor endothelium and epithelium remained healthy and the stroma and...
wound margins were devoid of inflammation. These grafts remained healthy clinically and histologically as long as 114 days. We have previously reported further evidence that these grafts did not undergo rejection. Recipient rats bearing these nonrejected grafts had no evidence of CTL activity, whereas recipients that had suffered a clinical rejection of the allograft had significant CTL activity.15 Twenty-two or 55% of these grafts were observed to undergo rejection.15 These allografts were rejected anywhere from 9 to 46 days with a mean rejection time of 17.1 days. This was significantly different from syngeneic controls ($P < 0.005$). These grafts also had minimal edema which cleared by day 7, but they then were noted to develop clinical signs of rejection as previously defined. These grafts were examined within 24 hr of rejection, 48–72 hr following rejection, at 10 days post-rejection, and several weeks after rejection. Figure 4 shows the clinical features of acute allogeneic graft rejection. Figures 5 and 6 demonstrate the histological features of a typical untreated allograft that underwent acute rejection at day 13 and was removed and fixed on day 14. Acute rejection was characterized by edema of the donor stroma which was infiltrated by a marked number of neutrophils, lymphocytes and macrophages (Fig. 5). In general the
stromal keratocytes appeared viable (Figs. 5, 6). There was neovascularization and inflammation of the recipient corneal bed (Fig. 6) with vessels extending across the wound for a short distance into the donor graft. There was a fibrinoid inflammatory reaction in the anterior chamber (Fig. 5). The iris vessels were markedly congested and there was a striking lymphocytic infiltrate in the iris stroma (Fig. 5). The donor endothelial and epithelial layers were swollen, necrotic and infiltrated by lymphocytes and macrophages (Fig. 6). As endothelial cells died they were replaced by fibroblasts.

When rejected allografts were examined several weeks following the acute inflammatory episode, the histological features were similar to those found in failed human grafts. Figure 7 represents a fully allogeneic cornea that clinically suffered rejection on day

Fig. 5. Acute rejection of allogeneic corneal graft (day 14). Donor stroma is markedly edematous with lymphocytes, macrophages and neutrophils. Keratocytes are viable (lower left). There is a fibrinoid inflammatory reaction in the anterior chamber. The iris stroma is infiltrated by lymphocytes and the vessels are congested (lower right). Anterior synechiae are artifactual (upper). (upper H&E, X33; lower left H&E, X225; lower right H&E X360).
Fig. 6. Higher power of Figure 5. Donor graft epithelium (upper left) is swollen, necrotic and infiltrated with inflammatory cells when compared to normal recipient corneal epithelium (upper right). Both donor and recipient corneal stroma is infiltrated by chronic round cells but, in addition, there is neovascularization of recipient stroma (arrowheads; upper left and right). Donor graft endothelium is swollen and necrotic (lower left) when compared to recipient endothelium (lower right). Lymphocytes adhere to the donor graft endothelium (arrows) while macrophages adhere to posterior cornea of both donor and recipient (all H&E, X900).

Latex bead-treated allografts: Seven days prior to grafting, shallow incisions were made in the donor cornea and a latex bead-saline solution was layered over the wound. When these corneas were studied histologically at day 7, prior to grafting, the incisions had resulted in a well-healed shallow stromal scar (corneal facet) which was filled in by corneal epithelium (Fig. 8, upper) and characterized by a few activated fibroblasts in the adjacent corneal stroma. Neovascularization and inflammatory cells such as macrophages, lymphocytes or neutrophils were never present. The latex beads were phagocytized by both epithelial cells and stromal keratocytes (Fig. 8, lower). Again, these latex beads resulted in the migration of Langerhans cells into the central cornea. These allografts contained donor Langerhans cells and were invariably rejected. Of 23 such grafts, 22 or 96% were rejected by a mean time of 11.8 days. This was significantly different from untreated allografts (P < 0.001). These grafts were also studied histologically within 24 hr of rejection, 48-72 hr following rejection, at 10 days post-rejection, and several weeks after clinical rejection. At the time of rejection, these grafts could not be distinguished from untreated rejecting allografts. Figure 9 demonstrates the histological appearance of a latex-treated allograft observed to undergo clinical rejection on day 13 and fixed and removed on day 15. The iris was congested and infiltrated with lymphocytes and there was a fibrinous inflammatory reaction in the anterior chamber. The stroma was again infiltrated with neutrophils, macrophages and lymphocytes. The endothelium was pyknotic with neutrophils and lymphocytes present. The epithelium also resembled that of untreated rejecting allografts. When examined several weeks post-rejection
these grafts also had thinned stroma that was relatively clear of cellular infiltrate, only fibroblasts replacing the endothelium, and thinned epithelium. Thus, even though these latex treated allografts produced marked DTH responsiveness in the host, there was no difference in the histologic picture of rejection when compared to untreated allografts.

Allografts placed on preimmune hosts: In Lewis rats that were initially grafted with WF skin and 4 weeks later grafted orthotopically with WF cornea the rejection of these corneal allografts was 100% (10 of 10 rats). The speed of rejection was also increased to a mean of 9.7 days. This was significantly different from untreated allografts ($P < 0.001$), but was not significantly different from latex-treated allografts. These grafts were also studied within 24 hr of rejection, 48–72 hr following rejection, at 10 days post-rejection, and several weeks post-rejection. The histological features of acute rejection of these grafts were markedly different from the other groups. Figures 10 and 11 show the histologic picture of an allograft placed on a preimmune host that underwent clinical rejection on day 10 and was fixed and removed on day 10. The anterior chamber in the majority of these grafts had no significant infiltrate. In addition, the iris and ciliary body did not display the congestion or inflammation noted in untreated and latex-treated rejecting allografts. The endothelium was again swollen and necrotic with attached lymphocytes and neutrophils (Fig. 10). Both the keratocytes and infiltrating inflammatory cells of the corneal stroma displayed pyknosis and karyorrhexis. The epithelium was edematous and necrotic with infiltrating lymphocytes and neutrophils. Several weeks following rejection, these grafts could not be reliably distinguished from rejected untreated allografts. The endothelium was replaced with fibroblasts, the stroma was hypocellular and thinned and the epithelium was thinned.

Discussion

We have reported the use of a rat model of orthotopic corneal transplantation to study the characteristics of rejection. The advantages of this model include the use of a well-characterized immune system and the ability to perform a true penetrating kerato-
plasty. We have previously reported the basic immunologic characteristics of this model including the incidence and speed of rejection of allografts, the development of cytotoxic lymphocytes by the recipient and the requirement for donor Langerhans cells to induce DTH responses in the recipient. We report here the histologic characteristics of this model. Although others have reported loss of each of the cellular components, there is considerable evidence that donor endothelium, keratocytes and epithelium can survive for prolonged periods of time in an orthotopic corneal transplant. Basu, Miller and Ormsby used sex chromatin as a marker to demonstrate that donor keratocytes could survive at least 3 months in the cat. Chi et al also used sex chromatin to demonstrate survival of donor endothelium in the rabbit. Hanna and Irwin and Polack et al used tritiated thymidine to show survival of keratocytes and endothelial cells. Khodadoust and Silverstein used methylene blue staining, histology and tritiated thymidine to...
Fig. 9. Acute rejection of latex bead-treated corneal graft (day 15). There is a fibrinoid inflammatory reaction in the anterior chamber (upper, H&E, X33). Corneal stroma is edematous with neutrophils, lymphocytes and macrophages. Iris is infiltrated by lymphocytes and the vessels are congested (lower left, X360). Corneal endothelial cells are swollen and necrotic with lymphocytes (arrowheads) and neutrophils attached. Keratocytes are viable (lower right, H&E, X900).

Further show the survival of donor epithelium in rabbits. In each of these studies, the precise genetic disparity between donor and recipient was unknown. In particular, the amount of difference in terms of the major histocompatibility complex was uncertain. The two strains of rat we have used differ at both class I and II MHC loci and at minor transplantation loci as well. They therefore represent the greatest transplantation barrier possible. We have shown that these fully allogeneic corneas can survive for as long as 114 days when grafted in an orthotopic position with the transplant sutures removed. These grafts have remained clear both clinically and histologically when compared to syngeneic grafts. There was no histologic indication of damage to either epithelium, keratocytes or endothelium. It is unlikely that this represents replacement of donor components with recipient endothelium or epithelium. Tuft et al. have reported that rat endothelium does have limited regenerative capacity but to repopulate an area the size of our graft would take a minimum of 30 days, based on their observations. Khodadoust and Silverstein also state that in rabbit cornea the required time to develop full thickness replacement of donor epithelium with recipient epithelium is on the order of 2 to 3 weeks. For these reasons, we believe that it is very unlikely that any rejection has occurred when the grafts appear healthy and free of any infiltrate or damage as early as 10 days and continue to remain free of inflammation both clinically and histologically. In addition, we have already reported that rats bearing these nonrejected allografts have no evidence.
of systemic sensitization as measured by CTL and DTH activity. The histologic picture confirmed the previous findings of Khodadoust and Silverstein. All three layers of the cornea in our model could be affected by the rejection process. Epithelium was drastically affected in preimmunized rats but was eventually affected even in untreated and latex treated grafts. Of interest are the findings of Treseler, Foulks and Sanfilippo, who used the various cellular components of rat cornea in a heterotopic grafting model. They demonstrated that rat endothelium or stroma alone when grafted onto a thoracic dermal bed could lead to the development of antigen specific CTL in the host. However, rat epithelium alone when grafted heterotopically did not induce CTL in the host. This may represent a unique characteristic of the afferent or sensitization portion of the immune response. Treseler et al have suggested that this finding is related to the absence of class II antigen in the epithelium. Our data would indicate that if the host is sensitized to the alloantigens by the presence of the other layers, the epithelium can serve as a target of the immune response. It is important to recall that our studies using this rat model were done without the use of any steroids. Despite this, the histologic findings were quite comparable to those of Polack in his review of human graft rejection. One exception was the presence of cells in the anterior chamber. This may have been due to the difference in species or the use of steroids in humans to suppress the rejection process.

Latex treatment of corneal allografts resulted in migration of donor Langerhans cells into the central cornea and had a remarkable effect on the rejection process. These corneas had both a higher incidence and speed of rejection and led to a higher level of host CTL responsiveness when compared to untreated central allografts. In addition, these donor Langerhans cells produced a striking DTH responsiveness in
the host that was absent in hosts bearing untreated allografts.\textsuperscript{13} Despite these significant immunologic effects, the histologic picture of these latex-treated grafts was not different from that seen with untreated corneal allografts. The epithelium, stroma and endothelium were quite comparable to that of the untreated rejecting allograft. This remarkable finding would suggest that the presence or lack of DTH responsiveness in the host has no effect on the mechanism of graft rejection. The higher rate of rejection of latex-treated grafts (96\%) compared to untreated grafts (55\%) may be partly explained by an increased immunogenicity of the former grafts in terms of CTL responses.\textsuperscript{15} Treseler and Sanfilippo\textsuperscript{25} have reported the presence of "a few scattered (Ia\textsuperscript{+}) positive cells" in the central stroma and "extremely rare" class II MHC antigen-positive cells in the central epithelium of PVG rats. It is possible that the presence of these few Ia\textsuperscript{+} dendritic cells in untreated rat allografts are the cause of the histologic similarity between untreated and latex-treated allografts undergoing rejection.

Preimmunization by skin grafting did have an effect on the histology of graft rejection. The cornea underwent a process not unlike ischemic necrosis, with cell death of stromal keratocytes and the infiltrating inflammatory cells as well. The epithelium was rapidly destroyed and appeared to be sloughed without replacement by recipient epithelium initially. These animals possess an immune system sensitized to the alloantigens, including DTH, CTL and antibody responses. This prior sensitization of the immune apparatus probably accounts for the rapid and complete rejection of these corneas. The host bearing a latex-treated graft had no prior sensitization and therefore rejection of these Langerhans cell-containing allografts still represents first set rejection. This would also help explain the similarity between untreated and latex-treated allografts when examined histologically. The presence of donor Langerhans cells had a dramatic effect on the rejection incidence and immunologic sequelae of rejection but was not sufficient to replicate the conditions of prior sensitization to the alloantigens.

In summary, we have described the histologic picture of fully allogeneic corneal transplantation in the inbred rat. We have shown that in just less than half of the cases all components of allogeneic cornea...
could survive for a prolonged period of time in an avascular cornea. We have also described the typical histologic pattern for the allografts that are rejected. We have reported that although donor Langerhans cells have an effect on graft outcome they do not appear to alter the usual histologic pattern of first set rejection. Preimmunization with skin grafts had a significant effect on rejection histology.

Key words: cornea, transplantation, pathology, rat, rejection

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References