Xenoreactive CD4+ T Cells and Acute Rejection of Orthotopic Guinea Pig Corneas in Mice

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PURPOSE. To explore immunologic issues involved in orthotopic corneal xenotransplantation in a discordant combination using guinea pigs as donors and mice as recipients.

METHODS. Two-millimeter-diameter guinea pig corneal buttons were transplanted into 1.5-mm-diameter graft beds on mouse corneas using 12 interrupted sutures. Eyelids were maintained occluded with tarsorrhaphy except at the times of clinical inspection. Grafts were considered to be rejected when the pupil margin was not visible clearly through the graft by slit-lamp microscopy.

RESULTS. Guinea pig corneas protected from desiccation by persistent tarsorrhaphy survived indefinitely in the eyes of C.B-17SCID mice but were rejected acutely (but not hyperacutely) in eyes of normal BALB/c and C57Bl/6 mice (median survival times, MST, 16 and 10 days, respectively). Graft survival was not extended in mice deficient in C3 gene (MST of 21 versus 17 days) and greatly extended in mice deficient in the CD4 gene (MST of 26 versus 9 days). Reconstitution of CD4 knock-out (KO) mice with CD4+ T cells promoted acute rejection of corneal xenografts.

CONCLUSIONS. Hyperacute rejection does not occur in guinea pig corneal xenografts in mouse eyes, indicating that corneal xenografts are less vulnerable to this type of rejection than other solid tissue xenografts. CD4+ T cells are the primary mediators of acute graft rejection, although complement may contribute in a minor way. Neither antibodies nor CD8+ T cells participate in acute graft rejection. Because guinea pig cornea grafts in eyes of CD4KO mice are rejected in a delayed fashion, other innate and/or adaptive immune effectors must also be able to cause rejection of orthotopic corneal xenografts. (Invest Ophthalmol Vis Sci. 2000;41:1827–1832)

Experiments with xenografts in laboratory animals have revealed that the barriers to success of xenografts significantly exceed the barriers to success of allografts. In experimental animals, organ xenografts, such as kidney and heart, typically suffer immune rejection that is exceedingly fast, often within minutes to hours. The rapidity of rejection reflects the fact that the sera of most species contain “natural” antibodies that react with antigenic epitopes on xenogeneic tissues. Species pairs in which the serum of each member contains these natural antibodies are termed “discordant.” Natural antibodies are usually directed at carbohydrate antigens, which are expressed on many different cell types, especially vascular endothelial cells. Antibodies, particularly IgM, and complement are the causes of this type of rejection. When hyperacute rejection is prevented experimentally, delayed xenograft rejection typically occurs within 2 to 3 days. Xenoreactive antibodies, antibodies plus natural killer (NK) cells through antibody-dependent cell-mediated cytotoxicity, NK cells alone, and macrophages have been implicated in this type of rejection. If xenografts avoid hyperacute and delayed rejection reactions, they are then vulnerable to cell-mediated rejection, in which T cells are responsible. Rejections due to this mechanism occur at or beyond 6 to 7 days after grafting.

There are features of the cornea that suggest that its fate as a xenograft might be different from that of other types of solid tissue grafts. First, the cornea is avascular, and, therefore, it may not be vulnerable to hyperacute rejection that occurs when natural antibodies bind to vascular endothelial cells. Second, the cornea forms the anterior surface of the anterior chamber, and the aqueous humor that fills the chamber contains several extremely potent anticomplementary activities. Even if natural antibodies were to bind to corneal endothelium, factors in aqueous humor may thwart complement activation. Third, the cornea is devoid of bone marrow–derived cells termed “passenger leukocytes.” In other solid tissue organs, dendritic cells and macrophages confer on the graft a high level of immunogenicity. In their absence, the ability of a graft to induce immunity to graft-derived antigens is reduced. Fourth, the constitutive expression of CD95 ligand on corneal endothelium has been revealed as a critical factor in the privileged survival of orthotopic corneal allografts in mice.

In the recent past, two groups of investigators have reported on the fate of orthotopic corneal xenografts in a discordant combination using rats as recipients. Whereas Larkin et al. observed rejection of corneal xenografts within 3 days and ascribed the mechanism of rejection to humoral antibodies, Ross et al. didn’t observe rejection until 7 to 9 days and reported indirect evidence that antibodies participated in the
1828 Tanaka et al.

Fate of Xenogeneic Corneal Grafts Placed Orthotopically in the Eyes of Severe Combined Immune Deficient Mice

Our first series of corneal transplants was performed in C.B-17SCID mice, a circumstance in which the recipient lacks adaptive immunity. When tarsorrhaphy was maintained through, all grafts survived beyond 8 weeks (n = 8). In Figure 1A, a SCID mouse eye containing a guinea pig cornea at 4 weeks after transplantation is displayed. The central portion is clear, and the pupil margin can be seen clearly through the graft. Because the guinea pig cornea is approximately three times thicker than the mouse cornea, the margins of the grafts usually became opaque, due to the relative inability of the attenuated endothelium at this point to maintain stromal de-turgescence. In Figure 1B, the histologic appearance of an H & E-stained section of grafted tissue at 8 weeks is displayed. The epithelium is intact, a continuous layer of endothelium lines the posterior surface of the graft, and the stroma is well-ordered in a lamellar array with few neo-vessel profiles and no identifiable infiltrating cells. These findings indicate that in the absence of an adaptive immune response, guinea pig cornea xenografts survive well when placed orthotopically in the eyes of mice.

Fate of Xenogeneic Corneal Grafts in the Eyes of Normal (Immunocompetent) Mice Eyes

BALB/c and C57BL/6 mice were used as recipients of guinea pig cornea grafts. The results displayed in Figure 2A show a Kaplan–Meier survival curve of guinea pig grafts. In BALB/c guinea pig cornea grafts. The results displayed in Figure 2A show a Kaplan–Meier survival curve of guinea pig grafts. In BALB/c
mice, the MST was 16 days, and in C57BL/6 mice the MST was 10 days. An H & E-stained histologic section of a rejected corneal xenograft at 12 days after grafting in a C57BL/6 mouse eye is displayed in Figure 2B. The graft stroma is extremely edematous and contains many infiltrating cells, but without evidence of extravasated erythrocytes. Neither an epithelium layer nor an endothelial layer can be identified.

**Pattern of Xenoreactive Antibody Formation after Orthotopic Corneal Xenografting in Normal Mice**

Xenoreactive (anti-guinea pig) antibodies, which are constitutively present in normal mice serum, were measured using flow cytometry. After xenografting, the constitutive levels of anti-guinea pig antibodies remained virtually constant throughout the 4-week observation period (data not shown). By contrast, anti-guinea pig IgG antibody levels rose significantly after grafting (at 3 weeks in BALB/c recipients and at 2 weeks in C57BL/6 recipients; data not shown). Thus, orthotopic guinea pig corneas make an impact on the recipient B cell system, leading to increased production of anti-guinea pig IgG antibodies at times that correlated with acute rejection of these grafts.

**Fate of Xenogeneic Corneal Grafts in Mice with Selected Genes Knocked Out:**

**μ Heavy Chain of Immunoglobulin, Third Component of Complement, β-2 Microglobulin, and CD4**

Mice deficient in the μ heavy chain of immunoglobulin gene (μ KO) lack B cells and the capacity to produce antibodies. These mice, plus their wild-type controls, served as recipients of orthotopic guinea pig cornea grafts. The MSTs of these grafts were determined and are presented in Table 1. Grafts in both groups of mice were rejected acutely, and there was no difference between the two groups. This result formally excludes antibodies as a requirement for acute rejection of orthotopic guinea pig cornea grafts.

Mice deficient in the third component of complement gene (C3KO) are unable to carry out complement activation through either the classic or alternative pathways. Guinea pig grafts were placed in the eyes of these mice and their wild-type controls. The MSTs of their respective graft survivals are presented in Table 1. Grafts in the eyes of C3KO mice survived somewhat longer (statistically significant) than grafts in the eyes of wild-type mice. Although the effect is modest, this finding implies that complement may contribute to corneal xenograft rejection.

**FIGURE 1.** (A) Clinical appearance of an accepted guinea pig cornea graft in the eye of a C.B-17SCID mouse at 4 weeks after transplantation. The central portion of the graft is clear (black arrows point to graft margin), and the pupil margin can be seen clearly behind (and through) the graft (white arrows point to pupil margin). (B) H & E-stained histologic section of an accepted guinea pig cornea grafted in the eye of a SCID mouse at 8 weeks. Epithelial and endothelial layers are intact; the stroma is well organized with lamellar arrays and contains few neovessels and no infiltrating cells. Magnification, ×100.

**FIGURE 2.** (A) Kaplan–Meier survival curve of guinea pig cornea grafts in eyes of BALB/c (□) and C57BL/6 (○) mice. MST of grafts in eyes of BALB/c mice is 16 days. MST of grafts in eyes of C57BL/6 mice is 10 days. (B) H & E-stained histologic section of guinea pig cornea graft in the eye of a C57BL/6 mouse at 12 days. Graft stroma is edematous and contains many infiltrating cells. No epithelial or endothelial layer is visible. Magnification, ×100.
Mice deficient in the β2 microglobulin gene (β2 µ KO) express class Ia and Ib molecules poorly, and these mice are deficient in class I-restricted CD8+ T cells and NK T cells. As the results presented in Table 1 reveal, guinea pig corneas were rejected acutely and at comparable rates in β2 µ KO mice and in their wild-type controls. At least with respect to acute xenograft rejection, these results indicate that CD8+ T cells and NK T cells are not required.

CD4KO mice lack class II-restricted CD4+ T cells, an important effector of solid tissue and cornea allografts. Mice of this type and their wild-type controls received orthotopic guinea pig cornea grafts. As the results presented in Table 1 reveal, grafts in the eyes of CD4KO mice survived significantly longer than grafts placed in the eyes of wild-type mice (P < 0.0001). For discussion purposes, we refer to the rapid rejection (within 2–3 weeks) of corneal xenografts in normal C57BL/6 and BALB/c mice as “acute” and the delayed rejection (beyond 3–6 weeks) observed in CD4KO mice as “chronic.” To explain the difference between acute and chronic rejection, we postulate that CD4+ T cells are the primary, if not the only, mediators of acute rejection of orthotopic guinea pig cornea grafts in mice.

Fate of Xenogeneic Corneal Grafts in the Eyes of CD4KO Mice Reconstituted with CD4+ T Cells

To test the hypothesis that CD4+ T cells are the mediators of acute corneal xenograft rejection in mice, a reconstitution experiment was performed. Guinea pig corneas were grafted into the eyes of CD4KO mice 1 day after these mice received, intravenously, 15 × 10⁶ CD4+ T cells from syngeneic donors with an intact CD4 gene. The results of this experiment are presented in Figure 3. Reconstituted CD4KO mice rejected guinea pig cornea grafts significantly more swiftly than did nonreconstituted CD4KO mice. This result confirms the key role played by CD4+ T cells in acute rejection of orthotopic guinea pig cornea xenografts in mice.

DISCUSSION

Although our experimental results are the first concerning the fate of orthotopic corneal xenografts in mice using a discordant combination, previous studies have described the fate of similar discordant xenografts in rat eyes. In fact, the published reports are in partial conflict. Whereas Larkin et al. reported that guinea pig corneas grafted to the eyes of rats were rejected in an extremely rapid fashion (2–3 days), Ross et al. reported that rats rejected similar orthotopic grafts between 6 and 9 days. The results of our experiments resemble the latter report, and we think that we may be able to explain the difference with the former. Because the guinea pig cornea is significantly thicker than the mouse cornea (and also thicker than the rat cornea), it is difficult for recipient eyelids to close completely over the grafted surface. As a consequence, the surface desiccates rapidly and the graft fails. By simply closing the eyelids after grafting by sutures (tarsorrhaphy), desiccation is avoided and the graft survives. In the absence of an adaptive immune response, as in SCID mice, guinea pig cornea grafts that were protected by tarsorrhaphy survived for a long time (at least 8 weeks).

We are confident that tarsorrhaphy has no unsuspected protective effect on the capacity of the immune system to destroy cornea grafts. We have conducted experiments with orthotopic corneal allografts that were protected by persistent tarsorrhaphy. We found that allografts placed in both normal and “high-risk” mouse eyes were rejected with the expected tempo and frequency of grafts unprotected by tarsorrhaphy (data not shown).

Our findings that orthotopic guinea pig xenografts survived in SCID mouse eyes beyond 8 weeks, but were rejected between 8 and 16 days in the eyes of normal C57BL/6 and BALB/c mice, indicate that cornea xenografts evoke a destructive adaptive immunity. A similar conclusion was reached by Yamagami et al. who reported on the fate of corneal xenografts in a discordant (rat to mouse) situation. Guinea pigs and mice are discordant, and mouse serum constitutively contains anti–guinea pig antibodies. Despite the finding that serum anti–guinea pig IgG titers rose in the sera of C57BL/6 and BALB/c recipients of guinea pig cornea mice (at about the time the orthotrophic grafts were rejected), the MST of cornea xenografts in mice genetically deficient in B cells and antibody formation was virtually identical with that of wild-type mice. Thus, we conclude that antibodies play little or no role in the process by which normal mice reject guinea pig cornea grafts acutely. Moreover, no hyperacute rejection (3 days or less) was observed in normal mice that received orthotopic corneal xenografts, indicating that the constitutive presence of preformed anti–guinea pig antibodies also has no deleterious
effect on graft survival. The utter failure of antibodies to influence graft outcome in mice probably relates to the blood-ocular barrier, which severely limits access of high-molecular-weight blood-borne molecules into the eye, and the intense anti-complementary properties of aqueous humor. Thus, the guinea pig cornea in the mouse eye differs from other solid tissue xenografts placed in mice in its virtual invulnerability to antibody-mediated rejection.

Rather, acute rejection of orthotopic guinea pig corneas in mice appears to be mediated primarily, if not exclusively, by CD4+ T cells. In the recent past, Takano and Williams have published indirect evidence that CD4+ T cells participate in rejection of corneal xenografts in rats. Pierson et al. reported a similar mechanism in the rejection of xenogeneic skin grafts in mice. Not only did we find that acute rejection was avoided by guinea pig grafts placed in the eyes of CD4KO mice but that reconstitution of these mice with normal CD4+ T cells restored the capacity to reject. No similar loss of capacity to reject grafts was observed in mice deficient in CD8+ (or NK) T cells. In mice, rejection of orthotopic skin xenografts is mediated largely by CD4+ T cells. When orthotopic corneal allografts have been studied in mice, CD4+ T cells have also been identified as the primary mediators of acute graft rejection. Moreover, the majority of effector T cells in this circumstance is activated via the so-called indirect pathway of allore cognition, which means that peptides from donor alloantigens are detected when presented in the context of recipient class I and II major histocompatibility complex molecules. We are currently testing whether recognition and rejection of guinea pig corneas in mice are similarly mediated by "indirect" xenoreactive CD4+ T cells.

Despite this emphasis on a key role for CD4+ T cells in acute rejection of corneal xenografts in mice, we are not discarding other effector mechanisms as possible contributors. Although guinea pig cornea grafts in the eyes of CD4KO mice were not rejected acutely, many of those grafted were eventually destroyed in a chronic fashion. We do not understand the pathogenesis of these delayed rejections. Several possibilities exist. First, CD4KO mice may, in principle, be slightly "leaky" (although we have no evidence to suggest that). If so, very small numbers of CD4+ T cells could emerge through time in these mice and accumulate in sufficient quantities to effect chronic graft rejection. Second, it is possible that CD8+ T cells participate in delayed, chronic, rejection. Our current results merely exclude these cells from participating in an important way in acute rejection. Third, the slight but significant prolongation in graft survival observed in the eyes of mice deficient in C3 may correlate with a role for complement in chronic graft rejection, perhaps through the antibody-independent, alternative, pathway. Finally, innate, rather than adaptive, immune effectors may be the culprits responsible for chronic xenograft rejection. Innate cells (NK cells, macrophages) have already been implicated in the rejection of other solid tissue and organ xenografts. Experiments to test this and other possibilities are currently under way.

We have initiated our studies on the immunobiology of orthotopic corneal xenografts because of unique properties of the cornea and the anterior chamber of the eye, which might mitigate the vulnerability of these grafts to rejection. Our results encourage further research in this area. The apparent invulnerability of corneal xenografts to antibody-mediated injury gives them a significant advantage over vascularized solid tissue xenografts. In addition, the key role played by CD4+ T cells in acute cornea xenograft rejection suggests that already established immunosuppressive regimens may exist that would be effective treatments. Topical steroid therapy has long been used to reverse orthotopic corneal allograft rejection in human beings and might be similarly effective in orthotopic corneal xenografts. In an abstract communication, She et al. reported that the survival of mouse corneas grafted to the eyes of rats was markedly enhanced by the induction of anterior chamber associated immune deviation to mouse alloantigens and by subconjunctival injection of dexamethasone. Experiments to test the effectiveness of topical and systemic immunosuppressive agents in the guinea pig to mouse orthotopic corneal xenograft model will be able to answer this important question.

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References