Fate of MHC-Matched Corneal Allografts in Th1-Deficient Hosts

Sylvia L. Hargrave, Christina Hay, Jessamee Mellon, Elizabeth Mayhew, and Jerry Y. Niederkorn

PURPOSE. To determine whether the Th1 cytokine, interferon (IFN)-γ, is necessary for corneal graft rejection.

METHODS. Full-thickness penetrating keratoplasties were performed in normal mice and in IFN-γ knockout (KO) mice.

RESULTS. Sixty-four percent of the MHC-mismatched corneal allografts were rejected in IFN-γ KO mice. By contrast, MHC-matched corneal allografts were rejected in 50% to 77% of the wild-type hosts, but were not rejected in any of the IFN-γ KO mice or the wild-type mice treated with anti-IFN-γ monoclonal antibody. Corneal graft rejection in IFN-γ-deficient hosts was characterized by an eosinophilic infiltrate compared with a mononuclear inflammatory infiltrate in normal mice.

CONCLUSIONS. IFN-γ is not necessary for the rejection of MHC-mismatched corneal grafts. However, IFN-γ and Th1 immune mechanisms are necessary for the rejection of MHC-matched corneal allografts that confront the host with foreign minor histocompatibility antigens. The immune response in atopic patients, as in IFN-γ KO mice, is characterized by cross-regulation of Th1 cytokines, such as IFN-γ. The present results indicate that MHC matching dramatically reduces the risk of corneal graft rejection when IFN-γ is depressed or absent. Thus, MHC matching may reduce the risk of corneal graft rejection in patients with atopic keratoconus. (Invest Ophthalmol Vis Sci. 2004;45:1188–1193) DOI:10.1167/iovs.03-0515

Keratoplasty is the oldest and most common form of solid tissue transplantation.1,2 The survival of corneal grafts in first-time recipients with no complications reaches 90% and makes keratoplasty one of the most successful categories of organ transplantation.3–6 Although the value of major histocompatibility complex (MHC) matching in most forms of organ transplantation, such as the kidney, is well established, considerable controversy surrounds the issue of whether MHC matching reduces the risk of corneal graft rejection.3–5,7,8 The Collaborative Corneal Transplantation Studies Group reported a multicenter clinical trial involving 419 patients and concluded that HLA matching did not significantly reduce the likelihood of corneal graft rejection.8 By contrast, Reinhard et al.7 examined the fate of corneal allografts performed on 398 patients at a single center and found that HLA matching produced a significant improvement in corneal graft survival. Völker-Dieben et al.3,6 followed 1681 keratoplasties performed by a single surgeon and found that HLA matching had a significant salutary effect on corneal graft survival. Recent studies in rodent models of keratoplasty have revealed the importance of minor histocompatibility (H) antigens as targets for the immune rejection of corneal allografts.9,10 In fact, it has been argued that minor-H antigens supersede MHC antigens as the most important barrier for corneal allograft survival.11

Immune rejection is the leading cause of corneal graft failure and remains the most important barrier to successful keratoplasty. The immune response to most antigens, including alloantigens, can follow one of two pathways. The first pathway involves the activation of a T lymphocyte population termed Th1 cells that have three distinct characteristics: expression of the CD4 surface determinant; production of interferon (IFN)-γ; and mediation of delayed-type hypersensitivity (DTH).11 A widely embraced paradigm in transplantation immunology proposes that most forms of organ graft rejection are mediated by Th1 cells. Studies in rodent models of keratoplasty have demonstrated a close correlation between the development of DTH to donor alloantigens and corneal allograft rejection.12,13 The notion that Th1 cells are pivotal for corneal graft rejection is further supported by the finding that in vivo depletion of CD4+ T cells causes a steep reduction in the immune rejection of orthotopic corneal allografts in mice and rats.14,15

Another subset of T cells, termed Th2 cells, secrete cytokines that cross-regulate Th1 cells and inhibit their mediator functions.11 Yamada et al.16 have shown that mice with immune responses that are manipulated to respond preferentially toward a Th2 pathway experience a sharp decrease in the production of the Th1 cytokine IFN-γ, and a greater than 50% reduction in corneal graft rejection. Collectively, these results suggest that blocking the expression of the Th1 cytokine IFN-γ may have a salutary effect on corneal graft survival. In this study, we examined the fate of corneal allografts in hosts with Th1 responses that were blocked by either disruption of the IFN-γ gene or by in vivo neutralization with anti-IFN-γ antibody.

MATERIALS AND METHODS

Mice

Female NZB (H-2b), BALB.B10 (H-2b), and B10.D2 (H-2d) mice were used as corneal allograft donors. BALB/C ByJ (H-2b) and BALB/c IFN-γ knockout (KO) mice served as graft recipients. All were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were treated according to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Orthotopic Corneal Transplantation and Clinical Evaluation

Corneal allografts (2.0 mm in diameter) were transplanted onto the right eyes of anesthetized mice by using a surgical procedure described by She et al.17 Mice were anesthetized systemically with an intraperie...
tional injection of pentobarbital sodium (1–2 mg/mouse; Abbott Laboratories, Chicago, IL) and topically with proparacaine (Alcon Laboratories, Tampa, FL). The donor graft was sewn into place using a 12 bite continuous 11-0 nylon running suture (Ethicon, Somerville, NJ). Topical antibiotic bacitracin/polymyxin B sulfate (Ocumycin; Bausch & Lomb, Tampa, FL) was applied immediately after surgery. No immunosuppressive drugs were used. Sutures were removed 7 days after surgery.

Grafted eyes were examined with a slit-lamp biomicroscope twice weekly throughout the study (50 days). Graft opacity was scored using a scale of 1 to 4 as previously described.18 Corneal grafts were considered rejected on two successive opacity scores of 3.

Antibody Treatment
A rat anti-mouse IFN-γ monoclonal antibody was isolated and purified from supernatants from the R4-6A2 hybridoma (American Type Culture Collection, Manassas, VA). This monoclonal antibody does not affect the activities of murine α- and β-interferons and has been used to neutralize murine IFN-γ in vivo.15 BALB/c mice were treated with intraperitoneal injections of 500 μg of anti-IFN-γ given twice per week for 7 weeks.

Delayed Type Hypersensitivity
DTH to donor alloantigens was measured using a conventional ear-swelling assay.22 Ears of experimental and control animals were measured with a Mitutoyo engineer’s micrometer immediately before ear challenge. An eliciting dose of 1 × 107 x-irradiated (3000 rad) lymph node cells suspended in 25 μL of Hank’s balanced salt solution (HBSS), was inoculated into the subcutaneous tissue of the right ear. The left ear served as a negative control and was not injected. Results are expressed as specific ear swelling = (24-hour measurement of experimental ear – 0-hour measurement of experimental ear) – (24-hour measurement of control ear – 0-hour measurement of control ear).

Skin Grafting
Full-thickness skin grafts were prepared as described elsewhere.23 Grafts were applied orthotopically and wrapped in plaster-of-Paris bandages. Casts were removed 7 days later and the grafts were inspected for evidence of rejection. Graft rejection was deemed complete when all remnants of surface epidermis were absent. The median survival times (MSTs) for the skin allografts were determined and compared for statistical significance with the Mann-Whitney test.

Latex Bead Treatment
Donor Langerhans cells (LCs) were induced to migrate centripetally from the limbus into the central cornea by instillation of sterile latex beads (1.0 μm; Sigma-Aldrich, St. Louis, MO) into shallow incisions in the corneal epithelium of normal B10.D2 donor mice.22 Ten days later, B10.D2 corneal allografts were prepared and transplanted to BALB/c IFN-γ KO recipients. Previous studies have shown that latex bead treatment induces the centripetal migration of donor LC, but does not elicit the recruitment of lymphocytes or granulocytes.22,23

Statistical Analysis
MSTs and mean rejection times (MRTs) were calculated for the various corneal allografts. The Mann-Whitney test determined the statistical significance in differences in MSTs. The differences in the incidences of rejection were evaluated by χ² analysis. Results for DTH assays were evaluated by Student’s t-test. Differences in all experiments were considered to be statistically significant at P < 0.05.

RESULTS
Rejection of MHC-Mismatched Corneal Allografts in the Absence of IFN-γ
One current paradigm in transplantation immunology proposes that corneal allograft rejection is a Th1-mediated process that is intimately associated with the production of IFN-γ.16 Accordingly, we examined the fate of BALB.B10 corneal allografts transplanted to wild-type and IFN-γ KO BALB/c hosts. BALB.B10 donors differ from the BALB/c recipients at the entire MHC, but are identical at all minor-H loci. The results show that 64% (14/22) of the BALB.B10 corneal allografts were rejected by wild-type BALB/c hosts and 71% (10/14) of the BALB/c IFN-γ KO hosts (Fig. 1). There was no significant difference in either the tempo or the incidence of graft rejection in the wild-type and IFN-γ KO mice. Thus, MHC-mismatched corneal allografts can undergo rejection in the absence of IFN-γ, the sine qua non for conventional Th1 immune responses.

Role of IFN-γ in the Rejection of MHC-Matched Corneal Allografts
Experiments were performed to determine whether IFN-γ and thus, Th1 responses, were required for the rejection of corneal allografts matched at the MHC, but mismatched at all minor-H loci. In the first experiment, NZB corneal grafts were transplanted to wild-type and IFN-γ KO BALB/c recipients. Although 77% (10/13) of the corneal allografts were rejected by the wild-type BALB/c hosts, none of the 27 NZB grafts was rejected in the IFN-γ KO hosts (Fig. 2A). Similar results were observed when B10.D2 corneal grafts were transplanted to similar hosts. In this donor-host combination 50% (7/14) of the B10.D2 corneal grafts underwent rejection in wild-type BALB/c hosts, whereas none of the corneal allografts was rejected in the 25 BALB/c IFN-γ KO hosts (Fig. 2B).

Gene disruption is sometimes associated with developmental anomalies that are not directly related to the gene that has been deleted.24 Therefore, it was important to confirm that depletion of IFN-γ by an alternative method would produce results similar to those of gene disruption. Wild-type BALB/c mice were treated twice weekly (500 μg/dose) with either anti-IFN-γ monoclonal antibody or an isotype control antibody. The results mimicked those in BALB/c IFN-γ KO mice—that is, only 1 of the 10 animals treated with anti-IFN-γ antibody rejected the B10.D2 corneal grafts during the 60-day observation period, whereas 50% (5/10) of the BALB/c mice treated with the isotype control antibody rejected their corneal grafts and 45% (5/11) of the untreated BALB/c mice rejected their B10.D2 corneal grafts (Fig. 2C).
Antigenicity and Immunogenicity of MHC-Matched Corneal Grafts for IFN-γ-Dependent Rejection

The remarkable absence of corneal graft rejection in IFN-γ KO mice and wild-type mice treated with anti-IFN-γ antibody, suggests that minor-H antigens are unable to provoke Th1-independent alloimmune responses (i.e., they are not immunogenic). Alternatively, it is possible that minor-H antigens are either not vulnerable or they are invisible to Th1-independent immune effector elements (i.e., they are not antigenic). To distinguish between these two possibilities, BALB/c that were depleted of IFN-γ by in vivo treatment with anti-IFN-γ antibody and bearing long-term (>60 days) B10.D2 corneal grafts were challenged with orthotopic B10.D2 skin grafts as a means of inducing a robust immune response to the B10 minor-H alloantigens. Full-thickness orthotopic B10.D2 skin allografts were also transplanted to normal BALB/c mice to determine the tempo of first-set B10.D2 skin allograft rejection in the BALB/c mouse. All the B10.D2 skin allografts were rejected by the BALB/c hosts bearing previously clear B10.D2 corneal allografts (MRT = 7 ± 1 days), which was significantly swifter \( (P = 0.006) \), albeit the same incidence of rejection (i.e., 100%), as B10.D2 skin allografts transplanted to untreated BALB/c mice (MRT = 10 ± 2 days). Six of the nine previously clear B10.D2 corneal allografts underwent immune rejection after challenge with B10.D2 skin allografts at day 60 (MRT for corneal grafts after skin grafting = 34 ± 0 days). The tempo of corneal graft rejection was not significantly different from the first-set rejection tempo for B10.D2 corneal allografts transplanted to untreated BALB/c mice (MRT = 35 ± 6 days; \( P > 0.05 \)). Histopathological examination of the rejected skin allografts and corneal allografts revealed inflammatory infiltrates that were characterized by a preponderance of eosinophils (Figs. 3, 4A). By contrast, B10.D2 corneal allografts that were rejected by wild-type BALB/c hosts were infiltrated with mononuclear cells with only occasional eosinophils (Fig. 4B). Although the MHC-matched, minor-H mismatched corneal allografts were not immunogenic, they were highly antigenic, as demonstrated by their prompt rejection once the hosts were immunized with an orthotopic skin graft bearing the donor’s minor-H alloantigens.

Effect of Donor-Derived LCs on Immunogenicity of MHC-Matched Corneal Allografts

Tissues such as the skin contain a contiguous network of LCs that serve as potent antigen presenting cells (APCs), which induce robust alloimmune responses by directly activating T cells. Class II-positive, B7-positive LCs are conspicuously absent from the central portion of the cornea that is normally used for transplantation.1,2 Although LCs are absent from the central cornea, their appearance can be induced by a variety of stimuli including the instillation of sterile latex beads.25 Experiments were performed to determine whether the presence of donor LCs would render MHC-matched corneal allografts immunogenic for Th1-independent immune responses. LC migration was induced by treating B10.D2 donor corneas with latex beads 10 days before transplanting the corneas to BALB/c IFN-γ KO hosts.30 Unlike normal, untreated B10.D2 corneal allografts, which did not undergo rejection in BALB/c IFN-γ KO mice, latex-bead-treated B10.D2 corneal allografts were rejected in 87% (13/15; MRT = 16 ± 7 days) of the BALB/c IFN-γ KO mice. Histopathological examination of the latex bead-treated corneal grafts revealed an inflammatory infiltrate composed of eosinophils (data not shown).
DTH Responses in IFN-γ-Deficient Mice

The close correlation between the appearance of DTH and corneal allograft rejection prompted us to examine the DTH responses in BALB/c IFN-γ KO mice that were grafted with latex-bead–treated (LC⁺) corneal allografts. DTH responses to B10.D2 alloantigens were evaluated by using a conventional ear-swelling assay 2 weeks after the corneal grafts underwent rejection. DTH responses to donor alloantigens were detected in both wild-type mice and IFN-γ KO mice that had rejected their corneal allografts (Fig. 5). However, histopathological examination of the DTH lesions revealed a mononuclear inflammatory infiltrate in the ears of wild-type mice, but a mixed infiltrate of eosinophils and mononuclear cells in the IFN-γ KO mice (Fig. 6). Thus, the appearance of DTH correlated closely with both Th1-dependent and -independent rejection of corneal allografts. However, there were important qualitative differences in the inflammatory cell populations that participated in DTH responses, depending on the presence or absence of IFN-γ.

**FIGURE 3.** Histopathological features of rejected B10.D2 skin allograft transplanted orthotopically to a BALB/c mouse treated with anti-IFN-γ antibody (500 µg of anti-IFN-γ given twice per week for 7 weeks) and bearing a clear, long-term B10.D2 corneal allograft (>60 days). Arrows: eosinophils. Original magnification, ×450.

**FIGURE 4.** Histopathological features of rejected B10.D2 corneal allografts. (A) Rejected B10.D2 corneal allograft in a BALB/c mouse treated with anti-IFN-γ antibody (500 µg of anti-IFN-γ given twice weekly for 7 weeks) and grafted with a B10.D2 skin allograft. Note eosinophils (arrows). (B) B10.D2 corneal allograft rejected by wild-type BALB/c mouse. Note mononuclear infiltrate. Original magnification, ×450.

**FIGURE 5.** DTH responses to B10.D2 alloantigens in BALB/c mice grafted orthotopically with Langerhans-cell–containing B10.D2 corneal allografts. (A) Wild-type BALB/c hosts and (B) IFN-γ KO BALB/c host. Subcutaneously (SC) immunized mice were injected with 1 × 10⁷ B10.D2 spleen cells 14 days before an ear-swelling assay. Ear swelling was assessed 14 days after corneal graft rejection. There were five animals in each group. Results are expressed as mean specific swelling ± SD *P = 0.001; **P = 0.0006.
The results reported herein provided three significant new insights: (1) MHC antigens on corneal allografts can be targeted by IFN-γ-independent immune mechanisms that culminate in a Th1-independent mechanism of graft rejection; (2) minor-H antigens on corneal allografts fail to induce IFN-γ-dependent, Th1-mediated rejection; and (3) the presence of donor LCs renders MHC-matched corneal allografts highly immunogenic and susceptible to IFN-γ-independent immune rejection directed at the donor’s minor-H alloantigens. The IFN-γ-independent immune rejection of corneal allografts is associated with an eosinophilic infiltrate that is reminiscent of inflammation that is seen during the immune rejection of fully allogeneic corneal allografts and the Th2-mediated rejection of skin and heart allografts.

The rejection of both MHC-matched and -mismatched corneal allografts in normal hosts was characterized by a lymphocytic infiltrate and the appearance of DTH to the donor’s alloantigens. This is consistent with a Th1 form of immune rejection. By contrast, in the absence of the Th1 cytokine, IFN-γ, a pronounced eosinophilic inflammatory infiltrate was present in the rejected corneal allografts and in the DTH lesions. We have previously shown that fully allogeneic corneal allografts, which express alien MHC plus multiple minor-H alloantigens, undergo a form of rejection that is characterized by an intense eosinophilic corneal infiltrate and a preferential lymphocyte secretion of Th2 cytokines. In the present study, we found a similar eosinophilic inflammation in both MHC-matched and -mismatched corneal allografts that underwent rejection. The preponderance of eosinophils in the rejected corneal allografts in IFN-γ KO hosts is consistent with a Th2 form of immune rejection that has been reported in other allograft models in which the host’s alloimmune response is biased toward a Th2 phenotype, either by disruption of the IFN-γ gene or by in vivo neutralization with anti-IFN-γ antibody. The eosinophilic infiltrates in the ear swelling biopsies specimens of IFN-γ KO mice were characteristic of DTH lesions in Th2-polarized hosts—a finding that further supports the notion that corneal allograft rejection in IFN-γ KO hosts is a Th2-mediated process.

Eosinophils have been implicated as effector cells in IFN-γ-independent allograft rejection. Eosinophils may mediate allograft rejection by secretion of cationic proteins such as major basic protein (MBP), eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase. Eosinophil MBP is found extensively in grafts rejected by Th2 cytokine-secreting CD4+ T cells and is known to be directly cytotoxic to corneal cells. Cytotoxicity, through nonspecific interactions with the anionically charged lipid membrane, causes membrane disruption and subsequent cytolysis. In addition to direct cellular cytotoxicity, MBP induces platelet and mast cell degranulation, thereby exacerbating the inflammatory response.

Our findings are in agreement with those of Yamada et al. who reported that skewing the host’s alloimmune responses in a Th2 direction results in a significant reduction in corneal graft rejection. Although both studies used B10.D2 corneal allografts transplanted to BALB/c mice with a Th2 bias, our allografts were placed into avascular graft beds, whereas Yamada et al. used high-risk BALB/c hosts that had prevascularized graft beds at the time the corneal grafts were transplanted. Such hosts express significantly different alloimmune responses than do “normal risk” hosts. Unlike hosts with prevascularized graft beds, the “high-risk” hosts have cytotoxic T-lymphocyte responses to donor alloantigens and display a remarkably higher incidence of corneal graft rejection.

The absence of rejection of MHC-matched, minor-H-mismatched B10.D2 corneal grafts in IFN-γ-depleted hosts was not due to the induction of immune tolerance; these hosts rejected B10.D2 skin grafts in a normal first-set fashion. Moreover, two thirds of the previously clear B10.D2 corneal grafts underwent accelerated rejection after the application of B10.D2 skin allografts. Thus, the weight of evidence supports the hypothesis that the absence of IFN-γ prevents the development of conventional Th1 alloimmune responses to minor-H antigens on orthotopic corneal allografts as a result of immunologic ignorance, rather than through the induction of immunologic tolerance.

The IFN-γ KO hosts used in our study can be compared with patients with atopic keratoconus in the sense that MHC-mismatched corneal allografts were transplanted into avascular graft beds in hosts who were biased to respond in a Th2-like manner. The BALB/c IFN-γ KO mouse, similar to the patient with atopic keratoconus, displays a higher than expected incidence of corneal graft rejection. In support of this is a limited preliminary study on rejected corneal allografts in patients with keratoconus that revealed the presence of eosinophils in rejected corneal allografts of atopic hosts compared with a mononuclear infiltrate in the nonatopic hosts. A larger number of patients with atopic keratoconus who have undergone keratoplasty are needed to establish the veracity of this hypothesis. Likewise, additional studies in different murine models of Th2-prone mice are also needed to confirm the initial findings in the IFN-γ-deficient, Th2-prone mice. The present findings indicate that MHC matching produces a dramatic improvement in corneal allograft survival in Th2-prone hosts. Thus, implementing MHC matching in patients with atopic keratoconus could have a significant impact in reducing the risk of immune rejection with no foreseeable added risk.
References


