CD8⁺ T Cell–Mediated Delayed Rejection of Orthotopic Guinea Pig Cornea Grafts in Mice Deficient in CD4⁺ T Cells

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Purpose. To determine the immunopathogenesis of delayed orthotopic corneal xenograft rejection in mice deficient in the xenoreactive CD4⁺ T cells that mediate acute rejection.

Methods. CB.17 SCID and BALB/c mice were used as recipients of orthotopic cornea grafts obtained from strain 13 guinea pigs. Before transplantation, SCID recipients, which do not normally reject guinea pig corneal grafts, were reconstituted with spleen cells (whole, CD4-depleted, CD4/CD8-depleted) or purified CD8⁺ T cells from normal BALB/c donors. Graft survival was assessed by clinical examination, and median survival times (MST) were calculated. Lymphocytes from mice that rejected guinea pig corneal grafts were analyzed in vitro for their capacity to respond to guinea pig xenantigens and to lyse guinea pig target cells.

Results. SCID mice reconstituted with whole spleen cells from BALB/c donors rejected guinea pig corneal grafts with a vigor identical with that of normal BALB/c mice (MST = 15 and 14 days, respectively), whereas SCID mice reconstituted with CD4-depleted BALB/c spleen cells rejected guinea pig corneal grafts in a delayed fashion (MST = 27 days), as did SCID mice reconstituted with purified CD8⁺ T cells from BALB/c donors. Although CD8⁺ T cells from rejector mice failed to lyse guinea pig target cells in vitro, the T cells proliferated and secreted IFN-γ in response to in vitro stimulation with guinea pig xenantigens.

Conclusions. Guinea pig cornea xenografts that avoid acute rejection in CD4⁺ T cell-depleted mice are vulnerable to rejection by CD8⁺ T cells. Effector CD8⁺ T cells destroy corneal xenografts through release of proinflammatory mediators (IFN-γ) rather than by cytotoxicity. (Invest Ophthalmol Vis Sci. 2003;44:175–182) DOI:10.1167/iovs.02-0050

In some parts of the world, shortages of human donor corneas lead to long waiting times for patients who need corneal transplants. It is possible that corneas harvested from suitable xenogeneic sources could be a reasonable alternative to this problem. However, the barriers to success of solid-tissue xenografts significantly exceed the barriers to success of allografts. The heightened barrier to successful xenografts results primarily from the presence in unimmunized recipients of so-called natural antibodies that bind to vascular endothelial cells of xenografts, triggering complement activation, clot formation, and hyperacute rejection. In the case of the cornea, distinctive features exist which partially exempt this tissue from this type of pathogenesis. First, the normal cornea is an avascular tissue. Second, the corneal endothelium constitutively expresses potent membrane-bound complement inhibitors. Third, the cornea forms the anterior wall of the anterior chamber—an immune-privileged site with a fluid (aqueous humor) that contains anticomplementary activity. Fourth, the cornea itself possesses inherent immune privilege, in part through the ability of its cells to secrete immunosuppressive factors and express CD95 ligand. Our laboratory has recently demonstrated that guinea pig corneas grafted heterotopically into the anterior chamber of eyes of normal mice whose sera naturally contain guinea pig-reactive antibodies are not vulnerable to hyperacute, antibody-mediated rejection, and that acute rejection of corneal xenografts occurs in mice genetically incapable of producing antibodies. These findings suggest that naturally occurring antibodies may not be an important barrier to successful orthotopic cornea grafting in humans.

However, the absence of antibody-mediated corneal xenograft rejection does not mean that mice are incapable of rejecting corneal xenografts. Tanaka et al. have reported that BALB/c and C57BL/6 mice reject guinea pig corneas in an acute manner, and they have provided convincing evidence that acute rejection is mediated by CD4⁺ xenoreactive T cells. Because the normal cornea lacks class II MHC-bearing antigen-presenting cells (APCs), it is not particularly surprising that the CD4⁺ T cells mediating acute corneal xenograft rejection are of the indirect type, and that is what has been found. That is, murine T cells appear to be incapable of recognizing and responding to MHC-encoded guinea pig xenantigens, but they readily respond to guinea pig xenantigens presented on murine APCs. Thus, rejection of orthotopic guinea pig corneal xenografts by mice is accomplished almost exclusively by indirect xenoreactive T cells, a situation virtually identical with the manner in which mice reject orthotopic corneal allografts.

When CD4⁺ T cells are eliminated from mice that receive orthotopic guinea pig cornea grafts, acute rejection is avoided, but the grafts eventually succumb to a delayed destructive inflammation that typically comes to completion by 30 days. Because immune deficient SCID mice have been found to be incapable of rejecting guinea pig cornea grafts, the delayed rejection observed in CD4⁺ T cell deficient mice strongly suggests that adaptive immune effectors are responsible. In the present study, we tested the hypothesis that CD4-independent CD8⁺ T cells are the mediators of delayed rejection of guinea pig corneal xenografts. The results support the validity of this hypothesis, and suggest that the effector CD8⁺ T cells cause graft rejection by releasing proinflammatory cytokines, such as IFN-γ, that promote macrophage-mediated graft destruction.
Reconstitution of SCID Mice with Normal Lymphoid Cell Populations

In some experiments, adult SCID mice were reconstituted with whole spleen cells (or fractions thereof) obtained from normal BALB/c. Subsequently, the reconstituted mice received orthotopic guinea pig corneal grafts. For reconstitution with whole spleen cells, BALB/c splenocytes, depleted of RBCs by lysis with Tris-NH$_4$-Cl, were suspended in Hank’s balanced salt solution (HBSS) and injected intravenously into CB17-SCID mice (one donor equivalent, approximately $45 \times 10^6$ cells/donor). For reconstitution with splenocytes depleted of CD4$^+$ T cells or CD4$^+$ + CD8$^+$ T cells, RBC-lysed splenocytes from BALB/c mice were incubated with anti-CD4 (GK1.5) alone, or in conjunction with anti-CD8 (2.43) antibodies (ascites fluid, a kind gift from Joan Stein Streilein, Schepens Eye Research Institute, Boston, MA) for 30 minutes on ice. washed twice with complete medium (CM) composed of RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin (all from BioWhittaker, Walkersville, MD), and 1 x 10$^5$ to 5 x 10$^5$ m-mercaptethanol (ME; Sigma Chemical Co., St. Louis, MO), supplemented with heat-inactivated 10% fetal calf serum (Sigma). The cells were then incubated with infant rabbit complement (Pel-Freeze Biologicals, Rogers, AR) for 30 minutes at room temperature, washed once, and resuspended in HBSS and injected (one donor equivalent) intravenously into SCID mice. $^{21,22}$ On the same day, the mice received 100 $\mu$L of anti-CD4 (GK1.5) antibodies (ascitic fluid diluted 1:2 with HBSS) injected intraperitoneally. Anti-CD4 antibodies were injected intraperitoneally at weekly intervals thereafter until the experiment came to completion. For reconstitution with purified CD8$^+$ T cells, cervical, inguinal, and mesenteric lymph nodes and spleens were obtained from naive BALB/c mice (two donors/recipient) and pressed through nylon mesh. After the RBCs were lysed, the suspension was passed through a T cell-enrichment column (Biotexx Laboratories, Inc., Houston, TX), followed by passage through a CD8$^+$ T cell-enrichment column (R&D Systems, Minneapolis, MN). The purity was 88% with no CD4$^+$ T cells (<0.1%). Purified CD8$^+$ T cells (approximately two donor equivalents, $4 \times 10^6$ cells/recipient) were injected intravenously into SCID mice. These recipient mice also received systemic treatment with anti CD4 antibody as described earlier. All reconstituted SCID mice received orthotopic guinea pig cornea transplants 1 day after reconstitution.

Orthotopic Corneal Xenografting

The detailed surgical procedure for performing orthotopic corneal xenotransplants in mice was previously reported.$^{17}$ Briefly, grafts were removed from donor guinea pig corneas by excision with a 2.0-mm-diameter trephine. The corneal tissue was then placed in HBSS until grafting. The graft bed was prepared by excising with Vannas scissors a 1.5-mm site from the central cornea of the right eye. The xenograft was placed in the recipient bed and secured with 10 interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX) juxtaposed to the epithelial surface of the recipient cornea. Antibiotic ointment was applied to the corneal surface, and the lids were closed with an 80 nylon tarsorrhaphy. Tarsorrhaphy was maintained (except for clinical inspection purposes) until graft rejection was documented. Corneal sutures were removed on day 8.

Assessment of Xenograft Survival

Corneal xenografts were evaluated by slit-lamp biomicroscopy three times a week. The day of rejection was defined when graft transparency was lost (i.e., the iris margin and iris structures were no longer visible clearly through the graft), and graft clarity never recovered subsequently.

In Vitro T-Cell Activation Assays

Cervical lymph nodes ipsilateral to the xenograft-containing eye were removed from BALB/c ($n = 6$) and reconstituted SCID mice ($n = 6$) 4 weeks after grafting—that is, at a time when the grafts were judged clinically to have been rejected. Lymph node cells from naive BALB/c mice were used as negative controls ($n = 6$). Single-cell suspensions prepared from lymph nodes of individual animals were used as responders ($5 \times 10^6$ cells/well) and added to x-irradiated (2000 R) 13 guinea pig stimulator spleen cells ($5 \times 10^6$ cells/well) in a final volume of 200 $\mu$L of culture medium. Triplicate cultures were prepared in 96-well flat-bottomed microculture plates (Corning, Corning, NY). The cultures were incubated at 37°C in an atmosphere of 5% CO$_2$ for 3 days. Eight hours before termination, the cultures were pulsed with 0.5 $\mu$Ci [H]$^3$-thymidine, and then harvested onto glass filters using an automated cell harvester (Tomtec, Orange, CT). Radioactivity was assessed by liquid scintillation spectrometry, and the amount expressed as counts per minute (cpm). For anti-CD4 or CD8 blocking experiments, anti-CD4 or anti-CD8 antibody ascitic fluid was added to the cultures at inception. In companion experiments, similar cultures were established and supernatants were collected at 96 hours and IFN-$\gamma$ levels were measured using enzyme-linked immunosorbent assay provided by a kit purchased from PharMingen. Coating antibody, detecting antibody and recombinant IFN-$\gamma$ as positive control were purchased from PharMingen. These experiments were repeated twice and similar results were obtained.

In Vitro Cytotoxicity Assay

To assess direct lymphocyte-mediated cytotoxicity by putative effector T cells obtained from SCID mice reconstituted with CD8$^+$ T cells or from BALB/c mice that had rejected guinea pig corneas, $^{51}$chromium (Cr)-release assays were performed on day 28 after corneal xenotransplantation. To create suitable target cells, 13 guinea pig spleen cells were co-cultured with 5 $\mu$L/g concanavalin A (ConA) for 3 days, then exposed to $^{51}$Cr for 2 hours. After washing, these cells served as targets of the putative effector T cells. Effector T cells were prepared from draining cervical lymph nodes obtained from mice that had rejected orthotopic corneal xenografts. These cells were restimulated in vitro by coculturing them with x-irradiated (2000 R) 15 guinea pig spleen cells for 3 days. Thereafter, the T cells were harvested from these cultures and added to $1 \times 10^7$ guinea pig Con A blast target cells in triplicate wells at ratios of effector-to-target cells of 6:1, 12:1, 25:1, 50:1, 75:1, and 100:1. Six wells containing medium and target cells were used to measure spontaneous release, and six wells containing 5
N HCl and target cells were used to measure maximal radioisotope release. After 4 hours' incubation at 37°C in an atmosphere of 5% CO₂, 25 μL of culture supernatant was removed from each well, and radioactivity was measured.²³

**Statistical Methods**

Statistical analysis of graft survival, enabling comparison of median survival times (MST), was performed using the Mantel-Cox rank test. Comparison of proliferation assay values was made by the Student's t-test. P < 0.05 was deemed significant.

**RESULTS**

The goal of these experiments was to create mice deficient in CD4⁺ T cells to determine the extent to which delayed rejection of orthotopic corneal xenografts is mediated by CD8⁺ T cells. Because SCID mice are incapable of rejecting guinea pig cornea grafts, our strategy was to reconstitute SCID mice with various splenic or lymph node cell populations and then to assess the capacity of these mice to reject orthotopic guinea pig cornea grafts.

**Fate of Guinea Cornea Grafts Placed in Eyes of SCID Mice Reconstituted with BALB/c Spleen Cells**

Adult SCID mice received intravenous infusions of cell suspensions prepared from spleens of normal BALB/c mice (one donor equivalent/recipient). One day later these mice received an orthotopic guinea pig cornea graft in the right eye. For comparison's sake, panels of normal BALB/c and SCID mice also received orthotopic guinea pig cornea grafts. One day later, peripheral blood was obtained from reconstituted SCID mice, as well as from normal SCID and BALB/c mice. Leukocytes in the blood samples were analyzed by flow cytometry for content of CD4⁺ and CD8⁺ T cells. As the results presented in Figure 1A reveal, peripheral blood from BALB/c mice contained easily identifiable populations of CD4⁺ and CD8⁺ T cells, whereas peripheral blood from 8-week-old SCID mice was devoid of these cells. Peripheral blood from SCID mice that received an infusion of BALB/c spleen cells 24 hours previously contained large numbers of both CD4⁺ and CD8⁺ T cells, indicating successful reconstitution of these adaptive immune cellular elements.

The survival of guinea pig cornea grafts was assessed clinically in reconstituted SCID mice and the relevant controls. The results presented in Figure 2A reveal that the survival of guinea pig cornea grafts was assessed clinically. The results presented in Figure 1B (compare the histogram at left with the histogram in middle), peripheral blood samples of SCID mice reconstituted with CD4⁺ T cells depleting spleen cells contained no CD4⁺ cells, but numerous CD8⁺ cells. SCID mice reconstituted in this fashion received orthotopic guinea pig cornea grafts 24 hours later, and the fate of these grafts was assessed clinically. The results presented in Figure 2B indicate that SCID mice reconstituted with CD4⁺ T cell-depleted spleen cells acquired the capacity to reject guinea pig cornea grafts, and that the tempo of rejection was prolonged compared to that of SCID mice reconstituted with whole spleen cells from BALB/c donors (MST = 27 days; MST = 14 days, P = 0.04). These results indicate that effectors present in normal BALB/c spleen cells depleting CD4⁺ T cells possess the capacity to reject guinea pig cornea grafts in the absence of CD4⁺ T cell help. The pattern of rejection observed in these mice is strikingly similar to that reported for CD4 knockout mice that received orthotopic guinea pig cornea grafts (MST = 27 days).¹⁷

**Fate of Guinea Cornea Grafts Placed in Eyes of SCID Mice Reconstituted with BALB/c Spleen Cells Depleted of CD4⁺ and CD8⁺ T Cells**

The previous result supports the hypothesis that CD8⁺ T cells, in the absence of CD4⁺ T cells, are capable of causing rejection of guinea pig cornea grafts, albeit in a delayed fashion. If the hypothesis is valid, it should be possible to eliminate the rejection of corneal xenografts in mice reconstituted with CD4⁺ T-cell-depleted spleen cells by also eliminating CD8⁺ T cells from the reconstituting inoculum. Accordingly, adult SCID mice received spleen cells depleted of CD4⁺ T cells; these mice were treated with both anti CD4 and anti CD8 antibodies plus complement. One day later the mice received orthotopic guinea pig cornea grafts, and immediately before the procedure peripheral blood was collected from these mice and analyzed for content of CD4⁺ and CD8⁺ T cells. As displayed in Figure 1B (compare histogram at left with the histogram at right), peripheral blood samples from these reconstituted mice contained no detectable CD4⁺ or CD8⁺ cells. Moreover, when the survival of guinea pig cornea grafts was assessed clinically (Fig. 2C), the most grafts were found to survive indefinitely (MST = 49 days). This result strongly suggests that the effector modality primarily responsible for delayed rejection of guinea pig grafts is a CD8⁺ T cell. However, this conclusion must be tempered by the observation that a minority (25%) of guinea pig cornea grafts were rejected in SCID mice reconstituted with BALB/c spleen cells depleted of both CD4⁺ and CD8⁺ cells.

**Fate of Guinea Cornea Grafts Placed in Eyes of SCID Mice Reconstituted with CD8⁺ T Cells Obtained from BALB/c Donors**

To confirm that CD8⁺ T cells on their own are capable of mounting rejection reactions that destroy orthotopic guinea pig cornea grafts, purified suspensions of CD8⁺ T cells were prepared from lymph nodes and spleens of normal BALB/c mice. As revealed in Figure 1C, peripheral blood of adult SCID
mice that received purified CD8<sup>+</sup> T cells one day previously contained large numbers of CD8<sup>+</sup> cells as detected by flow cytometry. Moreover, mice reconstituted in this fashion proved capable of rejecting orthotopic guinea pig cornea grafts (Fig. 2D). However, a minority of grafts was not rejected at all. On the one hand, these results permit us to conclude that CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup> T cell help are capable in their own right of developing xenograft immunity that leads to graft rejection. On the other hand, these results indicate that the efficiency of rejection mediated solely by CD8<sup>+</sup> T cells is less than that achieved with spleen cell suspensions depleted of CD4<sup>+</sup> T cells. This leads to the suspicion that yet another immune effector (non-CD4<sup>+</sup>, non-CD8<sup>+</sup>) lurks among spleen cells of normal BALB/c mice—cells capable of promoting re-

**Figure 1.** Flow cytometric evidence of reconstitution of SCID mice with CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells. Peripheral blood leukocytes obtained from SCID mice that received one day previously BALB/c (A) unfractionated spleen cells; (B) splenocytes depleted of CD4<sup>+</sup> or CD4<sup>+</sup>/CD8<sup>+</sup> T cells; and (C) purified lymph node and splenic CD8<sup>+</sup> T cells. The leukocytes were stained with antibodies directed at CD4 and CD8 molecules and then analyzed by flow cytometry. For positive and negative controls, peripheral blood leukocytes were obtained from normal BALB/c and untreated SCID mice. Histograms of individual assays are presented with CD4<sup>+</sup> cells along the y-axis and CD8<sup>+</sup> cells along the x-axis.
jection of corneal xenografts in the absence of either CD4$^+$ or CD8$^+$ T cells.

**Cytotoxic Capacity of CD8$^+$ T Cells Obtained from Mice that Rejected Guinea Pig Cornea Grafts**

A primary functional property of CD8$^+$ T cells is to recognize immunogenic peptides loaded onto class I major histocompatibility complex (MHC) molecules expressed on target cells as a prelude to delivery of a lytic signal that results in target cell death. We tested to determine whether cell-mediated cytotoxicity is the method by which xenoreactive CD8$^+$ T cells bring about cornea xenograft rejection in this system. Ipsilateral cervical lymph node cells were obtained from cervical lymph nodes draining eyes (of normal BALB/c recipients as well as SCID mice reconstituted with CD8$^+$ T cells) bearing rejected strain 13 guinea pig cornea grafts that had been in place for 28 days. The CD8$^+$ T cells were stimulated in vitro for 3 days by exposure to irradiated strain 13 guinea pig spleen cells. Thereafter, the responding T cells were placed in cytotoxicity assays in which 51Cr-labeled strain 13 guinea pig spleen cell Con A blasts were used as targets. Irrespective of the source of CD8$^+$ T cells, no evidence of cytotoxicity was observed (data not shown). These results suggest that direct cytotoxicity by effector CD8$^+$ T cells is not the mechanism by which cornea xenografts are rejected.

**Capacity of Guinea Pig Cells to Activate In Vitro CD8$^+$ T Cells Obtained from Mice that Have Rejected Guinea Pig Cornea Grafts**

In orthotopic corneal allografts in mice, rejection is achieved largely by CD4$^+$ T cells that recognize donor-derived alloantigens by the indirect pathway—that is, when processed and presented on recipient APCs that infiltrate the graft. This mechanism has been found to be responsible also for acute rejection of guinea pig cornea xenografts in mice. We next tested whether CD8$^+$ T cells from mice that had rejected guinea pig cornea grafts in the absence of CD4$^+$ T cells were activated in vitro by guinea pig spleen cells. The results are presented in Figures 3A and 3B. Spleen cells from mice that rejected strain 13 guinea pig cornea grafts activated CD8$^+$ T cells and produced IFN-$\gamma$ when stimulated with guinea pig spleen cells in vitro. Moreover, spleen cell suspensions from both types of rejector mice produced IFN-$\gamma$ when stimulated with guinea pig spleen cells in vitro. These results indicate that lymphoid cells obtained from mice that reject guinea pig corneas respond to guinea pig xenointigens in vitro by proliferating and by secreting the proinflammatory cytokine IFN-$\gamma$. 

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**Figure 2.** Survival patterns of strain 13 guinea pig corneas grafted orthotopically to eyes of SCID mice reconstituted with diverse BALB/c lymphoid cells. Guinea pig corneas were grafted orthotopically to SCID mice that were reconstituted one day previously with BALB/c (A) unfractionated spleen cells; (B) spleen cells depleted of CD4$^+$ T cells; (C) spleen cells depleted of CD4$^+$ T cells alone, or both CD4$^+$ and CD8$^+$ T cells; and (D) CD8$^+$ T cells purified from lymph node and spleen cell suspensions. Control panels of mice consisted of normal BALB/c and untreated SCID mice. Median survival times of xenografts (A) on BALB/c mice (14 d) and SCID mice reconstituted with whole splenocytes (15 d) are not significantly different; (B) on SCID mice reconstituted with unfractionated BALB/c splenocytes (15 d) versus CD4$^+$ T cell-depleted spleen cells (27 d) are significantly different ($P = 0.004$); (C) on SCID mice reconstituted with CD4$^+$ T cell-depleted splenocytes (27 d) versus CD4$^+$/CD8$^+$ T cell-depleted splenocytes (49 d) are significantly different ($P < 0.05$); (D) on SCID mice reconstituted with purified BALB/c CD8$^+$ T cells are significantly different from grafts placed on untreated SCID mice.
The results presented in Figure 3C indicate that the responding T cells in suspensions obtained from BALB/c mice that have rejected guinea pig corneas were almost exclusively CD8+. By contrast, the responding T cells in suspensions obtained from SCID mice reconstituted with CD8+ T cells that rejected guinea pig corneas were almost exclusively CD8-. Thus, in the absence of CD4+ T cells, xenoreactive CD8+ T cells acquire the capacity to become primed to guinea pig xenoor reactive xenografts to antibody mediated destruction.

**DISCUSSION**

The pathogenesis of orthotopic corneal xenografts is not completely understood. Early studies in which guinea pig corneas were transplanted orthotopically to rat eyes revealed a tempo of rejection that was not hyperacute, even though the investigators assumed that antibodies were responsible. More recently, Larkin et al. performed similar experiments and found that corneal xenografts were rejected in rat eyes within 2 to 3 days, strongly suggesting the importance of preformed antibodies in graft rejection. By contrast, our experiments over the past several years have come to a different conclusion, using a model in which guinea pig corneas are transplanted into eyes of mice, a species that is discordant—that is, the murine serum contains preformed anti-guinea pig antibodies. Our experiments have yielded two significant observations that have encouraged us to continue to study the immunopathogenesis of rejection of orthotopic corneal xenografts. First, guinea pig corneas grafted into the eyes of SCID mice survive indefinitely and maintain their clarity. This indicates that no important nonadaptive immune mechanism exists to limit the success of xenogeneic cornea transplants. Second, guinea pig corneal xenografts placed in eyes of mice with naturally occurring, preformed antibodies against guinea pig xenoor reactive xenografts suffer no evidence of hyperacute or delayed acute rejections of the antibody-mediated type. As mentioned previously, features that confer immune privilege on the cornea itself, as well as on the anterior chamber into which the graft is placed, undoubtedly account for the relative invulnerability of orthotopic corneal xenografts to antibody mediated destruction.

Encouraging as these observations are, they cannot obscure the important finding that guinea pig corneas placed in eyes of immunocompetent mice suffer acute rejection (within 8–20 days) that is mediated almost exclusively by CD4+ T cells that recognize guinea pig xenoantigens through the so-called indirect pathway. There is little evidence that immune privilege mitigates in any way the vigor and intensity of CD4+ T cell-mediated rejection of orthotopic guinea pig cornea grafts. Nor is there evidence that acute rejection of this type can be controlled by local or systemic immunosuppression with corticosteroids or cyclosporine A, agents that primarily target CD4+ T-cell–dependent immune rejection. As studies of this type proceed, it is important to determine whether immune effector modalities in addition to CD4+ T cells can also cause rejection of orthotopic guinea pig corneas in mice, and if so, what the nature of these effectors might be. The results reported here identify CD8+ T cells as capable of effecting destruction of guinea pig corneas grafted into the eyes of mice.

Whereas SCID mice reconstituted with BALB/c spleen cells rejected guinea pig corneas acutely (within 20 days) in a manner similar to intact BALB/c recipients, SCID mice reconstituted with BALB/c spleen cells depleted of CD4+ T cells or with purified CD8+ T cells from BALB/c donors rejected guinea pig corneas in a delayed fashion (between 20 and 40 days). The extent of proliferation was once again examined by measuring [3H]-thymidine incorporation and IFN-γ content of supernatants, respectively, in cultures containing CD8+ T cells that have rejected orthotopic guinea pig corneas acutely.

In the final set of experiments, we inquired into the CD4/CD8 phenotype of the T cells responding in the restimulation cultures. Cultures similar to those described were established with BALB/c spleen cells rejected guinea pig corneas acutely (within 20 days) in a manner similar to intact BALB/c recipients, SCID mice reconstituted with BALB/c spleen cells depleted of CD4+ T cells or with purified CD8+ T cells from BALB/c donors rejected guinea pig corneas in a delayed fashion (between 20 and 40 days). The extent of proliferation was once again examined by measuring [3H]-thymidine incorporation and IFN-γ content of supernatants, respectively, in cultures containing CD8+ T cells that have rejected orthotopic guinea pig corneas acutely.
A similar delayed rejection pattern was reported recently by Tanaka et al. who studied rejection of guinea pig cornea grafts in C57BL/6 mice in which the CD4 gene had been disrupted. Together these results permit the conclusion that CD8 T cells are sufficient, in the absence of CD4 T cells, to promote rejection of orthotopic guinea pig cornea grafts in mice.

One of the unexplained curiosities of immune rejection of orthotopic allogeneic cornea grafts is their relative resistance to rejection by direct alloreactive CD4 T and CD8 T cells—that is, the effector T cells that reject corneal allografs recognize donor allografts chiefly when processed and presented on APCs of recipient origin that infiltrate the graft. The same appears to be true for cornea xenografts. Tanaka et al. have recently reported that CD4 T cells mediated acute rejection of guinea pig cornea grafts exclusively by indirect xenoreactive T cells. Our current results extend this interpretation to the CD8 T cells that effect delayed rejection of guinea pig cornea grafts. We were unable to detect any guinea pig-specific cytolyltic T cells in mice that rejected guinea pig cornea grafts. However, we did detect the presence of both CD4 T and CD8 T cells that proliferated when stimulated in vitro with guinea pig spleen cells. Moreover, the responding T cells secreted significant amounts of IFN-γ. These results lead us to speculate that rejection of orthotopic guinea pig cornea grafts in mice is triggered by proinflammatory cytokines (such as IFN-γ) that are released from primed guinea pig xenotransplant-specific T cells that respond to guinea pig antigens displayed on recipient APCs that infiltrate the graft. A similar mechanism operates in the rejection of orthotopic corneal allografts in mice, and in this instance infiltrating recipient APCs have been well-documented to arrive in and on the graft shortly before and during the interval when the graft is rejected. To that end, Fox et al. showed that, once activated, macrophages can act as direct effectors and the responses of these cells is stronger against xenografts than alloantigens.

Although our experimental results are the first concerning the contribution of CD8 T cells to corneal xenograft rejection, other investigators examining other types of solid tissue xenografts have found a pathogenic role for CD8 T cells: porcine islet cell clusters to monkeys, or rat pancreas to mice, or hamster heart to rats. Most of these reports showed that CD8 T cells display cytotoxic activity for xenogeneic target cells, via perforin-mediated or Fas-Fasl interactions. Our results differ, in that we could find no evidence of direct cytolytic activity by in vivo primed, guinea pig xenogeneic-reactive CD8 T cells. A similar result was reported by Zhan et al. who showed that murine CD8 T cells could not lyse porcine splenocytes. Whether this deficiency represents a special property of murine CD8 T cells remains to be determined.

The precise mechanisms by which corneal xenografts in particular, and corneal allografts in general, are rejected remain elusive. Graft failure corresponds temporally with deterioration of function and eventual loss of corneal endothelium—the posterior layer of cells that is responsible for deturgescing the corneal stroma, thereby maintaining its clarity. Yet direct lysis of allogeneic or xenogeneic corneal endothelial cells by cytotoxic T cells does not appear to take place. In the case of corneal xenografts, we found no evidence of direct xenoreactive cytolytic CD8 T cells. In the case of corneal allografts, direct alloreactive CD8 T cells with cytolytic potential are formed, but appear to play no role in rejection. In the absence of direct cytotoxicity, immune rejection of allogeneic corneal grafts appears to be mediated by proinflammatory cytokines, especially IFN-γ, that are released by CD4 and/or CD8 T cells stimulated at the graft site by xenogeneic-presenting recipient APCs. Because activated macrophages and dendritic cells accumulate within the stroma and on the apical surface of corneal endothelium of rejecting grafts, the nonspecific destructive capacities of activated macrophages, perhaps through release of nitric oxide (NO) and reactive oxygen intermediates (ROI), are thought to be critical. Corneal endothelial cells are especially vulnerable to the deleterious effects of NO and ROI, and this may be why these grafts eventually fail.

Several findings reported here deserve special comment. First, CD8 T cells, on their own and without the aid of CD4 T cell help, are capable of causing the rejection of orthotopic guinea pig corneas in mice. This probably reflects the often-overlooked capacity of primed CD8 T cells to mediate reactions of the delayed hypersensitivity type. Second, even in the absence of CD4 and CD8 T cells, a small number of orthotopic corneal xenografts undergo rejection. Because untreated SCID mice never reject guinea pig cornea grafts, there must be another adaptive immune effector that can cause rejection on its own. Although we have no knowledge of the nature of this putative effector, we suspect that double negative T cells (perhaps cells using the γδ Tcr, or a subpopulation of NK T cells), and (2) B cells with an antibody product that could arm Fc receptor bearing leukocytes that mediate antibody-dependent cell mediated cytotoxicity. Our report that μ heavy-chain–deficient mice still reject guinea pig cornea grafts acutely merely reveals that antibodies are not involved in acute rejection. However, they could be involved in a more desultory rejection process such as that observed in mice deficient in both CD4 and CD8 T cells.

Finally, we would point out that the discovery of a potent, cornea xenograft-rejecting role for CD8 T cells is important on at least two accounts. On the first, CD8 T cells have been found to play virtually no role in rejection of orthotopic corneal allografts in mice. Thus, something is fundamentally different about corneal xenografts that renders these grafts, but not their allogeneic counterparts, vulnerable to rejection by CD8 T cells. On the second, the most immunosuppressive regimens now used to prevent rejection of solid tissue allografts in the clinic are directed at CD4 T cells, with little or no capacity to suppress CD8 T cell activity. Our current results indicate that immunosuppressive regimens that would be developed to suppress rejection of corneal xenografts must be effective at inhibiting both CD4 and CD8 T cells, or graft rejection will be the unfortunate outcome.

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