The Role of Cytotoxic T Lymphocytes in Corneal Allograft Rejection

Sushma Hegde[1,2] *and Jerry Y. Niederkorn*[2]

Purpose. Immunologic rejection constitutes a major barrier to the success of allogeneic corneal transplants, but the specific mediators and mechanisms of graft rejection are poorly understood. Several studies have implicated cytotoxic T-lymphocyte (CTL) responses, typically associated with $CD8^+$ T cells, in promoting corneal graft rejection. This study sought to test the hypothesis that CTLs are essential in promoting corneal graft rejection.

Methods. BALB/c donor corneas were grafted orthotopically onto C57BL/6, perforin knockout, or $CD8^+$ T-cell knockout mice. The tempo and incidence of graft rejection were observed for each group. In separate experiments, donor-specific CTL and delayed-type hypersensitivity (DTH) responses were tested at the time of graft rejection by a standard chromium release assay and an ear swelling assay, respectively.

Results. Perforin knockout and $CD8^+$ T-cell knockout mice were as effective as wild-type C57BL/6 control mice in rejecting BALB/c donor corneas. Furthermore, animals in all three groups were found to develop robust donor-specific DTH, not CTL, responses at the time of graft rejection. Histopathologically, the rejected corneas from all three groups contained a predominantly mononuclear cellular infiltrate.

Conclusions. This study rejects the hypothesis that $CD8^+$ CTLs are essential in promoting corneal graft rejection and instead further implicates donor-specific DTH reactions as the relevant immune response during graft failure. (*Invest Ophthalmol Vis Sci.* 2000;41:3341–3347)

The cornea currently enjoys the status of being the most commonly transplanted human tissue or organ, with more than 40,000 procedures/year in the United States alone.[1] Because neither HLA typing nor systemic immunosuppression (except in the case of high-risk individuals who have either received a previous corneal transplant or who have prevascularized graft beds) is performed routinely, it is remarkable that typical 2-year survival rates for initial grafts onto avascular graft beds are in excess of 90%.[1] Still, approximately 4000 corneal grafts fail each year in the United States because of immunologic rejection.[1]

The precise immunologic mechanisms leading to corneal graft rejection remain poorly understood. Although a large body of experimental evidence suggests that corneal allograft rejection is a cell-mediated process,[2–9] clinical studies have also implicated antibody.[10–15] Several investigators have studied the role played by cytotoxic T lymphocytes (CTLs) during orthotopic corneal allograft rejection, but there has been little consensus. Investigators have demonstrated the presence of donor-specific CTLs using the rat model of orthotopic corneal transplantation,[16–18] with clinical studies showing a correlation between corneal graft rejection and the presence of primed donor-specific CTLs.[19] Studies using the murine model of orthotopic corneal transplantation on the other hand have produced mixed results: some studies have suggested that CTLs do not play a role in corneal allograft rejection,[5,20,21] whereas others indicate that CTLs mediate an alloresponse to minor H alloantigens[22] and graft rejection in high-risk hosts.[21] The present study was conducted to investigate directly the role that CTLs play in corneal transplantation using a murine model of orthotopic corneal transplantation. Corneal grafts were performed on either perforin or $CD8^+$ T-cell–deficient hosts, both of which have impaired cytotoxic T-cell immune responses, and the fate of the corneal grafts as well as the donor-specific immune responses in these hosts were studied.

Methods

Animals

BALB/c ($H2^d$), C57BL/6 ($H2^b$), perforin knockout (C57BL/6-Pfp ⟨tm1Sdz⟩, $H2^b$), $CD8^+$T-cell knockout (C57BL/6-Cd8a ⟨tm1Mak⟩, $H2^b$), and C3H/Hej ($H2^k$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in pathogen-free animal facilities. The $CD8^+$ T-cell knockout mouse strain has been described previously as being deficient in $CD8^+$ T cells in peripheral lymphoid organs, and lacking CTLs to alloantigens as well as viral antigens. Helper T-cell development and function in this mouse strain is, however, comparable to normal wild-type controls.[23] By contrast, the perforin knockout mouse has been characterized as having

From the [1]Graduate Program in Immunology and [2]Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas.

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Corresponding author: Jerry Y. Niederkorn, Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9057.
jniede@mednet.swmed.edu

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normal numbers of $CD8^+$ T cells and NK cells but as having only 0% to 10% of the cytolytic response of wild-type controls to virus-infected or allogeneic target cells.[24] All animals used were female, 8 to 12 weeks in age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgical Technique

Full-thickness penetrating orthotopic corneal grafts were performed as previously described,[25] with a few modifications. Mice were anesthetized systemically with an intraperitoneal (i.p.) injection of 1.33×10^{-1} mg/kg of ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 6.68×10^{-3} mg/kg of xylazine (Bayer Corporation, Shawnee Mission, KS). Proparacaine HCl ophthalmic solution (USP 0.5%; Alcon Laboratories, Ft. Worth, TX) was used as a topical anesthetic. Donor grafts and recipient graft beds were scored with 2.5 and 2.0 mm trephines, respectively, and the corneas were excised with vannas scissors. Donor grafts were sewn into place using running 11-0 nylon sutures (Ethicon, Sommerville, NJ), and sutures were removed on day 7 posttransplantation. Topical antibiotic (Akorn, Decatur, IL) was applied immediately after surgery as well as immediately after removal of sutures. No immunosuppressive drugs were used either topically or systemically.

Clinical Evaluation of Grafted Corneas

Corneal grafts were examined two to three times a week with a slit-lamp biomicroscope (Carl Zeiss, Oberkochen, Germany). Graft opacity was scored using a scale of 1 to 3 as previously described.[21] Corneal grafts were considered rejected upon two successive scores of 3.

Cell Lines

Tissue-cultured BALB/c corneal epithelial and endothelial cells were used as targets for chromium release assays rather than the usual lymphoid cells because the corneal cells are the relevant target cells in vivo during corneal allograft rejection. Furthermore, it has been shown that the corneal epithelial and stromal cell layers express MHC class I antigens, with little to no expression on the corneal endothelial cell layer,[26,27] whereas lymphoid cells express high levels of MHC class I antigens. Cell cultures were established as described previously.[28] Briefly, cell cultures were established from freshly dissected corneal explants[29,30] and propagated in minimum essential medium (MEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT). Once primary cultures were established, the cells were immortalized with human papilloma virus genes E6 and E7, using the disabled recombinant retroviral vector *pLXSN16E6/E7*.[31] The transformed corneal cells proliferate indefinitely, maintaining their original morphologic characteristics and expressing the same histocompatibility antigens as their nontransformed counterparts.[32] Cell lines were maintained in complete MEM medium (BioWhittaker) containing 10% heat-inactivated FBS (HyClone Laboratories), 2 mM L-glutamine (BioWhittaker), 1 mM sodium pyruvate (BioWhittaker), 2 mM MEM vitamins (BioWhittaker), and 1% penicillin-streptomycin-fungizone solution (BioWhittaker).

Chromium Release Assay

A standard 4-hour ^{51}Cr release assay, as previously described,[25] was used to measure CTL activity in vitro. Briefly, single-cell suspensions of lymphocytes in complete RPMI-1640 medium (BioWhittaker) containing 10% heat-inactivated FBS (HyClone Laboratories), 2 mM L-glutamine (BioWhittaker), 1 mM sodium pyruvate (BioWhittaker), 1% penicillin-streptomycin-fungizone (BioWhittaker), 1% nonessential amino acids (BioWhittaker), 1% HEPES buffer (BioWhittaker), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) were prepared from various spleens and used as effector cells. Experimental and control effector lymphocytes were boosted in vitro for 96 hours at 37°C with γ-irradiated (3000 rad) donor strain stimulator spleen cells. The in vitro–boosted effector cells were washed and resuspended in complete RPMI medium. Effector cells were dispensed along with 2×10^4 ^{51}Cr-labeled donor strain corneal cells/well (corneal epithelial or corneal endothelial cells) in triplicate at several effector:target (E:T) ratios in a 96-well U-bottom microtiter plate (Corning Inc., Corning, NY), in a total volume of 200 μl/well. The plate was centrifuged at 500 rpm for 3 minutes before incubating at 37°C for 4 hours. The plate was then centrifuged at 800 rpm for 6 minutes before harvesting 100 μl of the supernatant from each well and counting on a gamma counter (Tracor Analytical, Atlanta, GA). Cytotoxicity was determined by the amount of ^{51}Cr released by the target cells, and the specific lysis was calculated as follows:

$$\frac{(\text{experimental cpm}) - (\text{spontaneous release cpm})}{(\text{maximum release cpm}) - (\text{spontaneous release cpm})} \times 100\%$$

DTH Assay

A standard ear swelling assay as described previously,[33] with a few modifications, was used to measure DTH to donor-specific alloantigens. Briefly, recipient mice were grafted with donor corneas 15 days before the DTH test. On day 15 posttransplantation, the right and left ear pinnae of grafted and control mice were measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) immediately before challenge. An eliciting dose of 4×10^6 γ-irradiated (3000 rad) donor strain, or third-party strain, splenocytes (erythrocytes were lysed before irradiation) suspended in 20 μl Hanks' balanced salt solution (HBSS; BioWhittaker) was injected into the right ear pinnae (experimental), whereas 20 μl HBSS was injected into the left ear pinnae (negative control). Both ear pinnae were measured 24 hours later, and the difference in ear pinnae size was used as a measure of DTH. Results were expressed as follows: Specific ear swelling = (24-hour measurement − 0-hour measurement for experimental ear) − (24-hour measurement − 0-hour measurement for control ear) $\times 10^{-4}$ inches.

Statistical Analysis

The Mann–Whitney U test was used to compare the median survival time (MST) between groups, whereas the graft rejection rates between groups were compared using the χ^2 test. The Student's t-test was used in all other cases. In each case, $P < 0.05$ was considered to be significant.

Results

The Fate of Corneal Allografts in Perforin or $CD8^+$ T-Cell–Deficient Hosts

A definitive experiment to determine the dependency of corneal graft rejection on the generation of donor-specific CTLs

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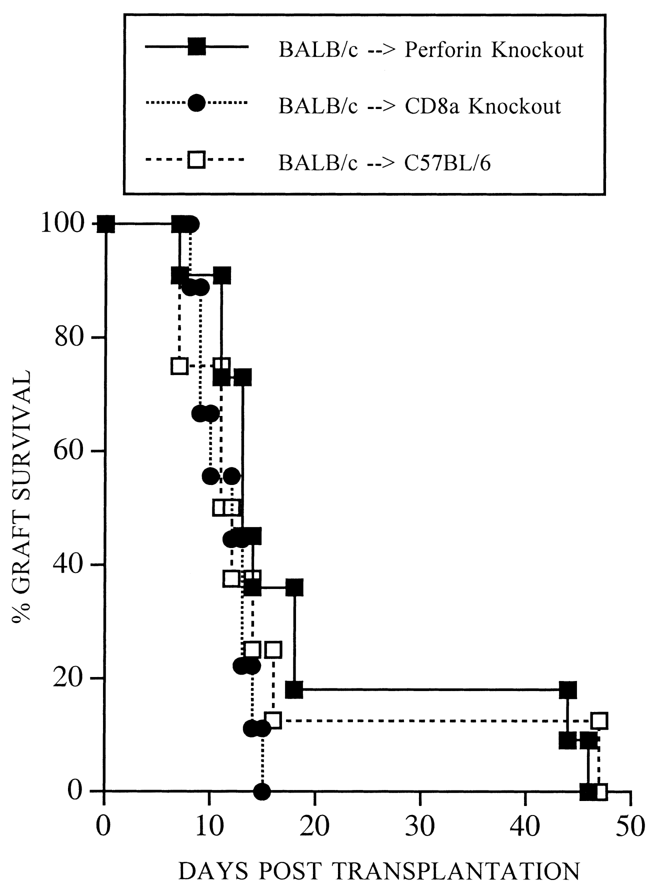

FIGURE 1. Corneal allograft rejection in CTL-deficient mice. BALB/c corneas were grafted orthotopically onto naïve perforin knockout mice (on a C57BL/6 background) ($n = 11$), naïve CD8$^+$ T-cell–deficient mice (on a C57BL/6 background) ($n = 9$), or onto wild-type controls (naïve C57BL/6J mice) ($n = 8$). The MST for BALB/c corneas grafted onto either perforin knockout hosts or CD8$^+$ T-cell–deficient hosts were not significantly different from wild-type C57BL/6 hosts ($P = 0.338$ and 0.847, respectively, by the Mann–Whitney U test).

would be to follow the fate of corneal allografts in mice with an impaired ability to generate CTLs. CTLs mediate their killing activity predominantly through the secretion of perforin granules, with the majority of CTLs exhibiting a CD8$^+$ phenotype. Thus, BALB/c corneal allografts were transplanted orthotopically onto either perforin-deficient mice (C57BL/6 background) or CD8$^+$ T-cell–deficient mice (C57BL/6 background). These donor/host combinations represent allodisparity at the entire MHC as well as multiple, minor H loci. We and others have found that normal C57BL/6 hosts reject 80% to 100% of orthotopic BALB/c corneal allografts.[34] Our results indicate that the incidence of rejection of BALB/c corneal allografts by either perforin-deficient or CD8$^+$ T-cell–deficient mice was essentially the same as in immunocompetent control mice (Fig. 1). Furthermore, the histopathologic features of rejected corneal allografts in both the perforin-deficient as well as the CD8$^+$ T-cell–deficient mice were very similar to grafts rejected by normal C57BL/6 hosts (Fig. 2). These combined results indicate that inactivation of the CTL arm of the immune response does not alter the course of corneal allograft rejection and thus argues against a role for CTL in corneal allograft rejection.

Donor-Specific Immune Responses in Perforin- or CD8$^+$ T-Cell–Deficient Hosts

Although our data suggested that CTLs do not play a role in corneal allograft rejection, they did not conclusively rule out the involvement of CTLs. A subset of CD4$^+$ T cells has been reported to have cytotoxic function,[35,36] and they could be playing a compensatory role in CD8$^+$ T-cell–deficient mice.

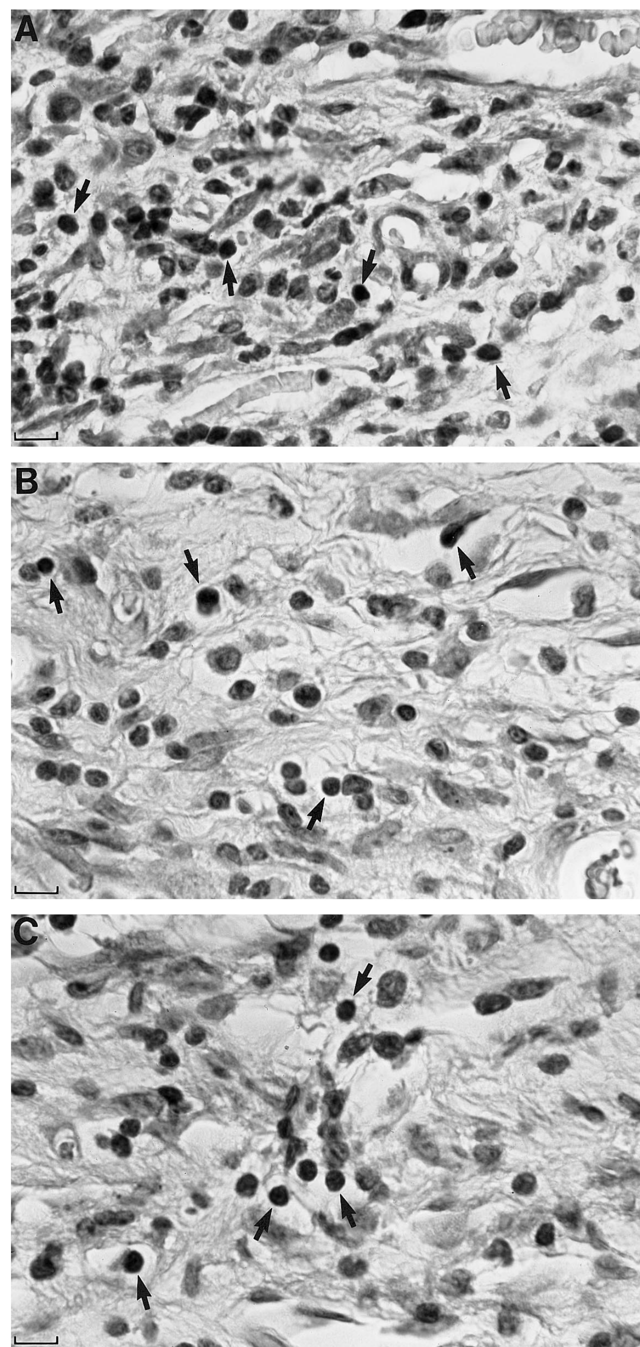

FIGURE 2. Histology of rejected corneal allografts. Wild-type C57BL/6J hosts (**A**), perforin knockout hosts (**B**), and CD8$^+$ T-cell–deficient hosts (**C**) all rejected BALB/c corneas with a predominantly mononuclear infiltrate (*arrows*) within the stroma typical of cell-mediated rejection. Hematoxylin and eosin staining was performed on all sections; bar, 20 μm.

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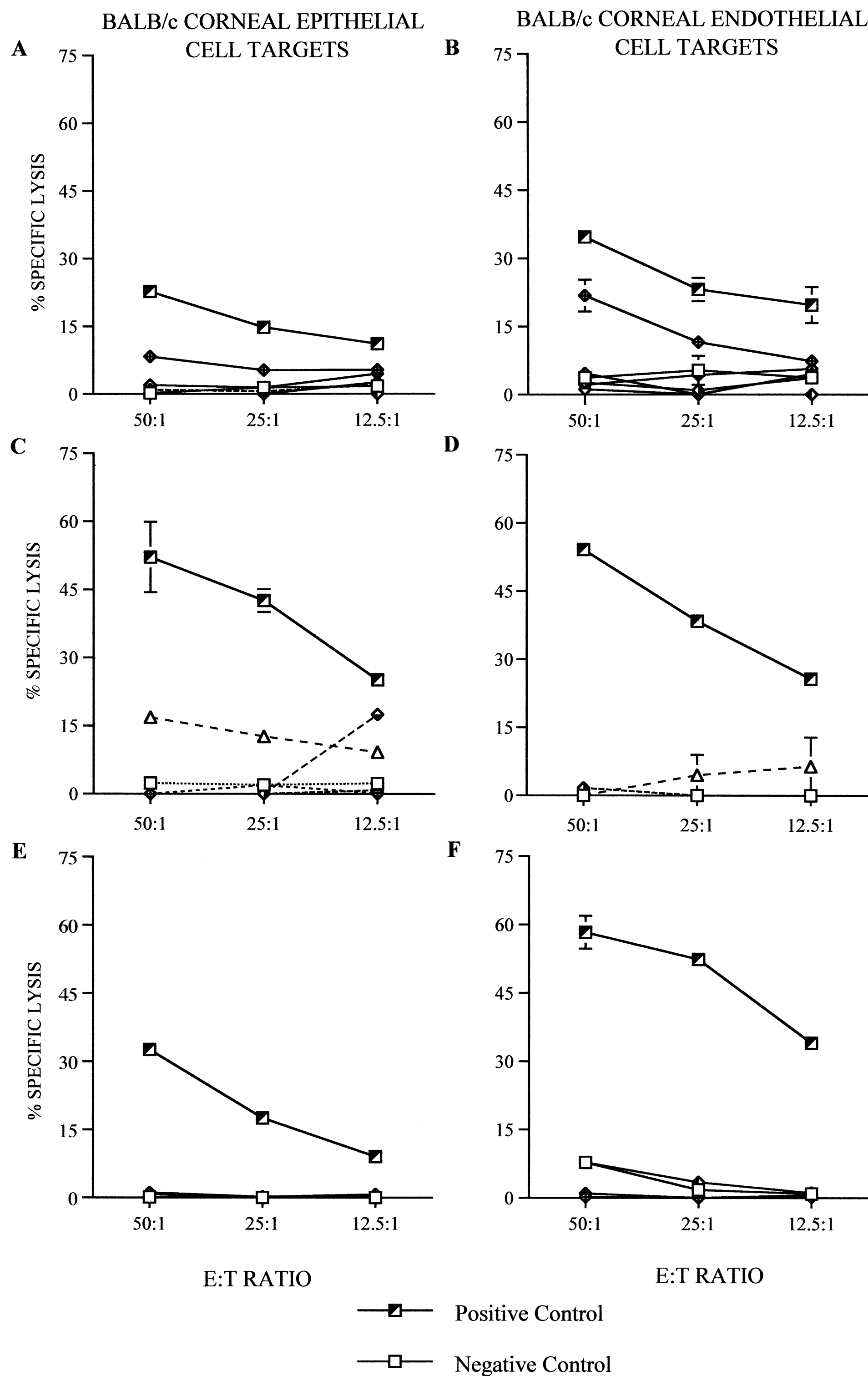

FIGURE 3. Evaluation of donor-specific CTL responses during corneal allograft rejection. Wild-type C57BL/6J hosts (**A**, **B**; $n = 5$), perforin knockout hosts (**C**, **D**; $n = 6$) and $CD8^+$ T-cell–deficient hosts (**E**, **F**; $n = 3$) were tested for donor-specific CTL responses 15 days after receiving an orthotopic BALB/c corneal graft, the MRT for each donor/host combination. Splenocytes from individually grafted animals were incubated with ^{51}Cr-labeled BALB/c corneal epithelial (**A**, **C**, **E**) or corneal endothelial (**B**, **D**, **F**) target cells. For each experiment, the positive control consisted of splenocytes harvested from a wild-type C57BL/6 mouse 10 days after immunization with 10^7 BALB/c splenocytes together with CFA, whereas the negative control consisted of splenocytes harvested from a naïve wild-type C57BL/6 mouse. Each datum point represents the mean percent specific lysis for triplicate wells at a given E:T ratio ± SEM.

Furthermore, although perforin secretion is the predominant mechanism used by CTLs to mediate their killing action, Fas–Fas ligand–triggered killing is an alternative mechanism that could be used by CTLs in perforin-deficient mice.[37] As further corroboration for our earlier data, we evaluated donor-specific immune responses in both perforin-deficient as well as $CD8^+$ T-cell–deficient hosts that had been transplanted with BALB/c corneal allografts. Donor-specific immune responses were evaluated at day 15 posttransplantation, which was within a few days of the median rejection times (MRTs) of BALB/c corneal allografts by both perforin- as well as $CD8^+$ T-cell–deficient mice (12 and 13 days posttransplantation, respectively). The MRT of BALB/c corneal allografts by immunocompetent C57BL/6 was 11.5 days posttransplantation.

We first evaluated donor-specific CTL responses using a standard 4-hour chromium release assay. As a positive control

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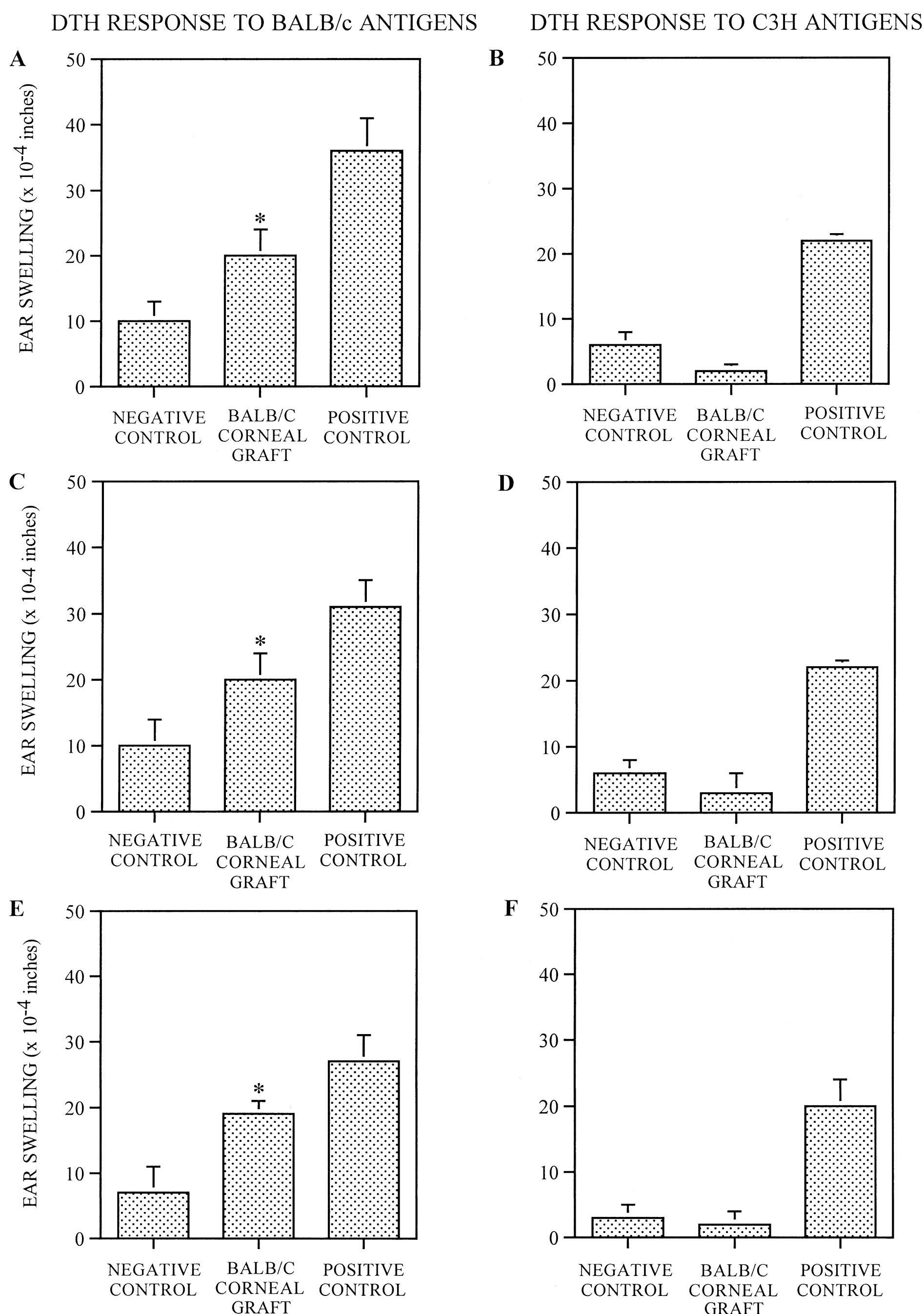

FIGURE 4. Evaluation of donor-specific DTH responses during corneal allograft rejection. Wild-type C57BL/6J hosts (**A**, **B**), perforin knockout hosts (**C**, **D**) and $CD8^+$ T-cell–deficient hosts (**E**, **F**) were evaluated for their ability to generate donor-specific DTH responses at day 15 posttransplantation, the MRT for this donor/host combination. Separate groups of animals were set up to test donor-specific DTH responses to BALB/c alloantigens (**A**, **C**, **E**) or third-party C3H/Hej alloantigens (**B**, **D**, **F**). Specific ear swelling responses were determined at 24 hours using a micrometer. Animals in the positive control groups were immunized with 10^7 BALB/c splenocytes (**A**, **C**, **E**) or 10^7 C3H/Hej splenocytes (**B**, **D**, **F**) together with CFA 10 days before testing for DTH responses. Animals in the negative control groups were naive C57BL/6 mice. Each bar represents the mean ear swelling of five animals ± SEM ($^*P < 0.05$).

for the assays we used a panel of wild-type C57BL/6 mice immunized against BALB/c donor antigens 8 to 10 days before the day of the assay, whereas a panel of naïve C57BL/6 mice was used as a negative control. The results are shown in Figure 3, and they indicate that by day 15 posttransplantation, none of the grafted animals had mounted a significant donor-specific CTL response directed against corneal epithelial target cells and only one animal demonstrated CTL activity against host corneal endothelial cells. Similar results were found using pooled lymph node cells as effector cells (data not shown).

We then evaluated donor-specific DTH responses using a standard ear swelling assay. For each experiment we used a panel of C57BL/6 mice immunized against BALB/c donor antigens as a positive control and a panel of naïve C57BL/6 mice as a negative control. The results are shown in Figure 4. The donor-specific DTH responses for the corneal graft recipients in each of the experimental groups (Figs. 4A, 4C, 4E) were significantly greater than the respective negative controls. These data indicate that while corneal allografts are rejected in the absence of any detectable donor-specific CTL response, they are rejected in the presence of a strong measurable donor-specific DTH response.

DISCUSSION

In spite of almost 50 years of research on animal models of keratoplasty, the precise immunologic mechanisms of corneal

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graft rejection remain a mystery. The seminal studies of Maumenee[38] provided the first evidence that the immune system might contribute to corneal graft failure. In a series of studies using a rabbit model of keratoplasty, Silverstein and Khodadoust[39] demonstrated that corneal graft rejection was a cell-mediated phenomenon that could be adoptively transferred to naive hosts. These studies also revealed that all three layers of the corneal graft could undergo independent immunologic destruction. Moreover, the role of lymphocytes in corneal graft rejection was supported by the appearance of the so-called "epithelial rejection line," which was characterized by a discrete zone of dead and dying epithelial cells surrounded by leukocytes, in front of which were apparently normal donor epithelium and behind which was a thin layer of dead donor epithelial cells.[40] Such piecemeal necrosis is consistent with the pattern of rejection one might observe if corneal graft rejection were mediated by CTLs, that is, CTL-mediated killing is contact dependent and occurs in a piecemeal fashion, one cell at a time.

The development of the rat model of penetrating keratoplasty by Williams and Coster in 1985[41] and the mouse model by She and coworkers in 1990[42] created new tools for examining the role of CTLs in the rejection of orthotopic corneal allografts. Studies in both the rat and mouse models of corneal transplantation demonstrated a correlation between the appearance of donor-specific CTLs and corneal graft rejection.[17,18,21,43] However, subsequent studies in mice demonstrated a relationship between the appearance of DTH responses to donor minor histocompatibility antigens and the rejection of orthotopic corneal allografts.[44] Moreover, in vivo depletion of $CD8^+$ T cells with monoclonal antibody failed to significantly enhance corneal graft survival in mice and thus cast doubt on the importance of conventional CTLs in corneal graft rejection.[5] In the present study, we took advantage of two different gene knockout mice to evaluate the role of perforin- and $CD8^+$ T-cell–dependent mechanisms in the rejection of orthotopic corneal allografts.

Our results suggest that CTLs do not play a role in the rejection of MHC and minor H mismatched corneal grafts. The ability of both perforin- as well as $CD8^+$ T-cell–deficient hosts to reject donor corneas as effectively as wild-type controls indicated that conventional CTLs are not essential for corneal allograft rejection. Still, this did not rule out the possibility that compensatory immune responses were operating in the absence of donor-specific CTL responses. However, histologic examination of rejected corneas indicated no significant differences in the histopathologic features of corneas rejected by either perforin- or $CD8^+$ T-cell–deficient hosts and wild-type controls; in all histologic sections studied, a predominantly mononuclear infiltrate was observed.

A subset of $CD4^+$ T cells has been reported to have cytotoxic function,[35,36] and thus, it was conceivable that they were acting to promote corneal graft rejection in the $CD8^+$ T-cell–deficient mice. Furthermore, although perforin secretion is the predominant mechanism used by CTLs to mediate their killing action, Fas-triggered killing could have been used as an alternative mechanism by CTLs in the perforin-deficient mice to induce graft rejection.[37] A functional study of the donor-specific immune responses in both the perforin- as well as $CD8^+$ T-cell–deficient mice demonstrated that corneal allograft rejection occurred in the absence of any detectable donor-specific CTL responses but coincided with the presence of a robust donor-specific DTH response. These data suggested that donor-specific CTL responses were not essential for the rejection of orthotopic corneal allografts and that the relevant immune response during graft rejection was a donor-specific DTH response. We suspect that $CD4^+$ T cells, the primary mediators of DTH responses, are the crucial effector cells mediating corneal allograft rejection. Experiments are currently in progress to elucidate the role of $CD4^+$ T cells in corneal allograft rejection, using a mouse model of orthotopic corneal allograft rejection.

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