T-Cell–Mediated Immune Responses in Alloepithelial Rejection after Murine Keratoepithelioplasty

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PURPOSE. To evaluate the role of delayed-type hypersensitivity (DTH) and cytotoxic T-lymphocyte (CTL) responses on alloepithelial rejection in a murine keratoepithelioplasty model.

METHODS. C3H/He mouse corneal lenticules were grafted around the limbus in BALB/c mice, and alloepithelial rejection was assessed by microscopic evaluation. The relation between rejection scores and DTH or CTL responses to donor antigens was assessed by Spearman correlation analysis. Suppression of DTH responses by induction of anterior chamber–associated immune deviation (ACAID) was used to evaluate the contribution of DTH responses to allograft rejection. CTL responses were evaluated by in vitro and in vivo depletion of CD4$^+$ or CD8$^+$ cells.

RESULTS. DTH responses, which developed 2 weeks postoperatively, correlated significantly with rejection scores (correlation coefficient $r = 0.55$). ACAID induction by anterior chamber inoculation of C3H/He splenocytes significantly suppressed allospecific DTH responses and alloepithelial rejection. While allospecific CTL responses also developed 2 weeks postoperatively and increased by 4 weeks, CTL responses did not exhibit positive correlation with rejection scores ($r = -0.36$, $P = 0.076$). The CTL responses were mediated exclusively by CD8$^+$ cells. Although in vivo depletion of CD8$^+$ cells abolished the induction of CTL responses, it did not prevent allograft rejection. Immunohistochemistry showed infiltration of CD4$^+$ and CD8$^+$ cells into the alloepithelium. Positive staining for interferon γ but not interleukin 4 further implicated the participation of a DTH response.

CONCLUSIONS. Allograft rejection after keratoepithelioplasty appears to be mediated primarily by DTH responses, with CTL responses playing only a minor role perhaps in modifying the development of rejection. (*Invest Ophthalmol Vis Sci.* 1999;40:2590–2597)

Orthotopic corneal transplantation enjoys a remarkably low rate of rejection when the allograft is placed in a nonvascularized corneal bed. However, for the treatment of chronic ocular surface disorders, such as those associated with chemical burns, Stevens–Johnson syndrome, or Mooren's ulcer, corneal transplantation has met with only limited success. Reconstruction of the ocular surface using keratoepithelioplasty (KEP) or limbal cell transplantation, both of which can supply healthy corneal epithelial cells, has been somewhat more successful.[1–4] Keratoepithelioplasty in which donor corneal grafts with alloepithelium are secured circumferentially around the recipient limbus exhibits a distinct clinical course and immunopathogenesis. Initially, the corneal surface becomes reepithelialized by donor-derived epithelium from the grafted lenticules.[5] After complete reepithelialization, epithelial type rejection is observed in addition to episodes of rejection against the grafted lenticules, and attentive postoperative care with long-term administration of topical immunosuppressives and use of bandage soft contact lenses is necessary to maintain the reconstructed ocular surface.[1–3]

Allograft rejection in corneal transplantation is believed to develop via a T-cell–mediated immune response. Specifically, delayed-type hypersensitivity (DTH) appears to play a dominant role in corneal allograft rejection as shown by several studies in the murine model of penetrating keratoplasty (PKP).[6–8] Moreover, Joo and colleagues[7] reported no significant difference in activity of cytotoxic T lymphocytes (CTL) between mice with accepted versus rejected corneas, suggesting that CTL responses may not play an important role in allograft rejection. > Yet, other studies report that allospecific CTLs do develop in rats with rejected corneas, but not in rats with accepted corneas.[7,10] Therefore, it remains unclear as to whether both DTH and CTL immune effector mechanisms participate in allograft rejection in rodent orthotopic PKP models.

However, the immune effector mechanisms involved in KEP likely differ from PKP for several reasons. First, rejection in KEP occurs mainly against the corneal epithelium, and does not occur against the endothelium. Second, grafts are secured around the limbus, which has abundant Langerhans cells allowing for greater host recognition of the grafts. Third, since graft components are separated from the recipient's anterior chamber, anterior chamber-associated immune deviation (ACAID) is not induced by the surgery itself. In contrast, in PKP, the DTH response is impaired in recipients with accepted corneal allografts, and ACAID is induced by the grafted corneal button itself.[6]

The murine KEP model we have developed is useful for investigating the role of DTH in orthotopic corneal transplantation, because it can directly examine the contribution of the DTH response without the overlaying influence of ACAID. [5] We previously reported that anterior chamber priming by alloantigens before grafting induced suppression of the DTH response and reduction of alloepithelial rejection in this murine KEP model with minor disparate grafting. [11] This suggested that DTH participates in alloepithelial rejection after KEP, although the role of other effector mechanisms was not examined. In the present study, we evaluated the contribution of both DTH and CTL responses to the immunopathogenesis of alloepithelial rejection after KEP, with examination of the role of specific T cell subsets using in vitro and in vivo depletion.

MATERIALS AND METHODS

Animals

Adult female BALB/c ($H-2^d$) and C3H/He ($H-2^k$) mice, disparate at major and multiple minor histocompatibility loci, were obtained from Japan SLC (Osaka, Japan). Animals were handled in accordance with the Association for Research in Vision and Ophthalmology Resolution for the Use of Animals in Ophthalmic and Vision Research.

Mouse KEP and Clinical Evaluation

The method of KEP grafting has been described previously. [5] In brief, full thickness cornea without scleral tissue was collected by excision along the limbus of the donor eye and cut into four 1.0-mm × 2.5-mm lenticules. Under anesthesia, the recipient corneal epithelium was scraped off completely, and adjacent limbal conjunctiva was removed circumferentially. Three donor corneal lenticules were secured around the recipient corneal limbus with two interrupted 11-0 nylon sutures (Alcon Surgical, Dallas, TX). Alloepithelial rejection was evaluated under an operating microscope and graded using previously established criteria, based on degree of corneal edema or opacity (0–4) and neovascularization (0–4). The rejection score was calculated as the sum of the edema/opacity score and the neovascularization score (0–8). Eyes with technical failures (such as anterior chamber perforation, hyphema, cataract, or postoperative infection) were excluded from the study.

Assay for DTH Reaction and Induction of Anterior Chamber–Associated Immune Deviation

At the indicated days after KEP, right ears were challenged using intrapinnal injection of C3H/He splenocytes (1.5×10^6 cells in 20 μl phosphate-buffered saline [PBS]) that had been lethally irradiated by cesium 137 (3000 cGy). PBS was injected into control left ears. Ear swelling was measured at 48 hours after antigen challenge using a micrometer (Mitutoyo, Tokyo, Japan). DTH responses were expressed as specific ear swelling = (48-hour measurement of right ear − 0-hour measurement of right ear) − (48-hour measurement of left ear − 0-hour measurement of left ear).

ACAID was induced by inoculation into the left anterior chamber of allogeneic C3H/He splenocytes (3.5×10^5 cells in 5 μl PBS) that had been lethally irradiated (3000 cGy). KEP was performed on the right eye 7 days after ACAID induction. KEP recipients inoculated with irradiated syngeneic BALB/c splenocytes served as negative controls.

Cell-Mediated Cytotoxicity

Spleens removed from BALB/c mice were processed into single cell suspensions and resuspended at a concentration of 6×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, and 0.1 mM nonessential amino acids. These cells were stimulated with irradiated C3H/He spleen cells (6×10^6 cells/ml) in vitro for 5 days. After this in vitro boosting, 100-μl aliquots of the splenocyte effector cell suspensions were added to round-bottomed microtiter plates at various concentrations. [51]Chromium-labeled target cells were added to the wells in triplicate to produce effector-to-target ratios (E/T ratios) ranging from 100/1 to 12.5/1 and incubated at 37°C for 4 hours. The target cells used were RDM-4 ($H-2^k$) for allogeneic CTLs, P815($H-2^d$) for syngeneic controls, and YAC-1 for measurement of natural killer (NK) activity. After centrifugation, 100 μl of each supernatant was collected, and counts per minute (cpm) were measured by a gamma counter. Specific cytotoxicity of each sample was calculated by the following formula: % specific lysis = [(experimental cpm − spontaneous cpm)/(maximum release cpm − spontaneous cpm)]× 100. Maximum ^{51}Cr release was determined by measuring the released amount into the medium by ^{51}Cr-labeled target cells incubated with 2% Triton X-100.

In Vitro Depletion of T-Cell Subsets

Before specific cytotoxic assay, splenocyte suspensions stimulated in vitro by irradiated C3H/He spleen cells for 5 days were incubated in diluted anti-L3T4 ascites fluid (50×), anti–Lyt-2.2 antibody (500×; Cedarlane, Hornby, Ontario, Canada), or anti–asialo GM1 antibody (50×; Wako, Osaka, Japan) on ice for 1 hour. Splenocytes were then resuspended in a 1:10 ratio with diluted rabbit complement (Cedarlane) and incubated for 1 hour at 37°C. Depletion of specific T-cell subsets was confirmed by FACS analysis (Becton Dickinson, Bedford, MA). Direct fluorescein staining was used to detect L3T4$^+$ or Lyt-2$^+$ cells using fluorescein isothiocyanate (FITC)–conjugated rat anti–mouse CD4 antibody (Serotec, Oxford, UK) or FITC-conjugated rat anti–mouse Lyt-2 antibody (Cedarlane). The depleted effector cells were used for the cytotoxic assay.

Preparation of Monoclonal Antibodies and In Vivo Depletion of T-Cell Subsets

The GK 1.5 hybridoma cell line (anti-L3T4) and the 53.6.72 hybridoma cell line (anti–Lyt-2) were propagated in nude mice, with monoclonal antibodies isolated from ascites fluids. Antibodies were purified using E-Z-SEP and Hi Trap Q ion exchange chromatography (Pharmacia Biotech, Piscataway, NJ). Protein concentrations were determined using BCA reagents (Pierce, Rockford, IL).

In vivo depletion of T-cell subsets was conducted by intraperitoneal injection of monoclonal antibodies, starting on 3 consecutive days 1 week before the KEP procedure, and continued twice a week until the end of the observation period at a dose of 0.1 mg/mouse for L3T4 antibody and 0.2 mg/mouse for Lyt-2 antibody, respectively. Specific depletion was confirmed by FACS analysis of splenocytes obtained from injected mice.

Immunohistochemistry

Eyes were enucleated at day 3, day 7, day 14, day 21, 1 month, 2 months, and 4 months after KEP. Eyes used for Thy1.2 staining were fixed in paraformaldehyde-lysine-periodate (PLP) fixative for 24 hours at 4°C, then embedded in paraffin, cut into 6-μm–thick
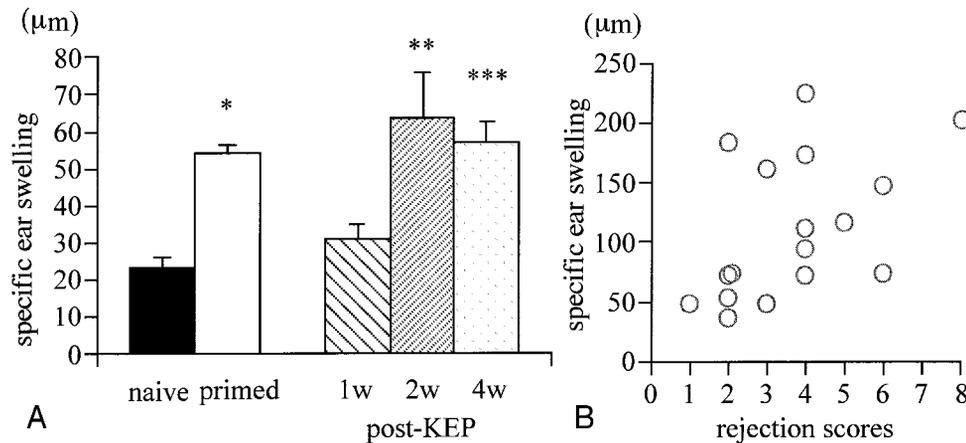
FIGURE 1. (A) Induction of DTH response against alloantigen from C3H/He donor after KEP. Ear swelling responses at 48 hours were measured using a micrometer. Primed mice were immunized with 7×10^6 C3H/He splenocytes in CFA. *Bars* represent the mean ear swelling of 6 mice per group $\pm$ SEM ($*P < 0.00001$, $**P < 0.05$, $***P < 0.0005$). (B) Correlation between DTH response to C3H/He donor antigen and rejection scores 2 weeks after KEP. Spearman correlation analysis showed significant correlation ($r = 0.55$, $P < 0.05$, $n = 18$).

sections, and deparaffinized. Other samples were prepared in 7-μm-thick frozen sections and fixed in acetone at $-20°$C.

The sections were then incubated with 0.3% H_2O_2 for 30 minutes to quench endogenous peroxidase activity. Immunohistochemistry was performed using a streptavidin–horseradish peroxidase system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. The primary antibodies used were rat anti–mouse Thy1.2 (Becton Dickinson), rat anti–mouse L3T4 (GK1.5), rat anti–mouse Lyt-2 (53.6.72), rat anti–mouse interferon γ (RMMG-1), and rat anti–mouse interleukin (IL)-4 (Pharmingen, San Diego, CA). Sections were developed with diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) and counterstained with methyl green.

Statistical Analysis

Data were analyzed using the two-tailed Student's *t*-test or Mann–Whitney U test as appropriate, and differences were considered significant for $P < 0.05$.

RESULTS

Correlation of Alloepithelial Rejection with DTH

The combination of BALB/c recipient and C3H/He graft, disparate in both major and multiple minor histocompatibility antigens, was used for analysis. We have observed that this combination causes more rapid and aggressive rejection than grafts disparate in minor antigens only, although 100% rejection is observed in both models. Clinical observation of epithelial responses was described in detail in our previous report.[5] Briefly, after a short interval of clear cornea with complete reepithelialization, epithelial rejection, characterized by an elevated edematous line on the host corneal bed, commences from the periphery to the central cornea. Edema of the donor lenticules and host cornea is observed in the initial phase of rejection with the edema/opacity score peaking 1 week postoperatively in our setting of C3H/He donors and BALB/c recipients. Sequentially, new vessels invade the lenticules and host cornea, followed by a decrease in the edema/opacity score. Massive invasion and engorgement of new vessels in the lenti-

cules and host cornea peak 2 weeks postoperatively, along with culmination of rejection and neovascularization scores. Further episodes of milder rejection then occur, after which corneal opacification and new vessels are observed to regress, and the rejected lenticules and host cornea are replaced by scarring and neovascularization.

To determine the role of the DTH response in this rejection, we investigated whether the pattern of DTH induction correlates with graft rejection. DTH responses were measured at weekly intervals after KEP. The kinetics of the DTH response against alloantigen from the C3H/He donor is shown in Figure 1A. An alloantigen-specific DTH response initially appeared at 1 week ($P = 0.12$) and peaked at approximately 2 weeks ($P < 0.05$). This DTH response was found to persist at least through s postoperative week 4 ($P < 0.0005$). To estimate the contribution of DTH to allograft rejection, Spearman correlation analysis was used to analyze the data at 2 weeks (Fig. 1B). Specific ear swelling of the grafted mice were found to correlate significantly with rejection scores ($r = 0.55$, $P < 0.05$).

Correlation of Alloepithelial Rejection with CTL Response

We next examined whether a CTL response against alloepithelium was induced in our KEP model, as assessed by the ability of splenocytes to lyse ^{51}Cr-labeled target cells. RDM-4 tumor cells (H-2K^k) were used as allospecific target cells. CTL assays were performed at weekly intervals after KEP (Fig. 2A), and vigorous allospecific CTL responses were detected 2 weeks postoperatively. This was compared with CTL responses after skin transplantation, a known inducer of strong allospecific killer activity (Fig. 2B). Two weeks after grafting from the C3H/He donor to the BALB/c recipient, although tail skin grafting (3 mm $\times$ 5 mm oval) elicited significant CTL responses, KEP grafting induced even more vigorous responses.

Allospecificity of the killer activity was confirmed using P815 mastcytoma cells (DBA2 origin, H-2D^d) as target cells. Activated splenocytes from BALB/c recipients exhibited no cytolytic activity against syngeneic P-815 cells (data not shown).
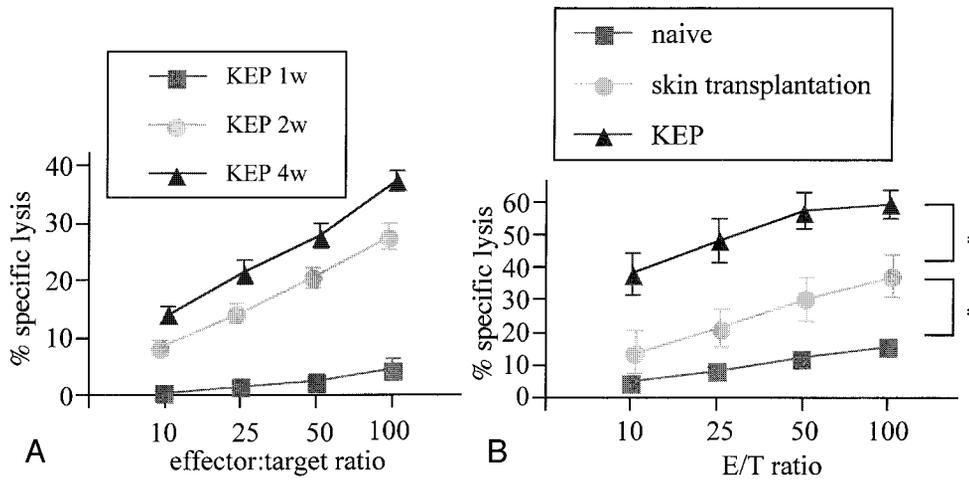
FIGURE 2. (A) Kinetics of allospecific CTL response against C3H/He donor antigen. Splenocytes from mice at various postoperative weeks were tested for their ability to lyse ^{51}Cr-labeled RDM-4 target cells. *Bars* represent mean specific lysis of 4 mice per group ± SEM. (B) Comparison of CTL responses between KEP and skin grafting from C3H/He donors. Splenocytes from recipients 2 weeks postoperatively for their ability to lyse ^{51}Cr-labeled RDM-4 target cells. *Bars* represent mean specific lysis of 4 mice per group ± SEM (*$P < 0.05$). ET, effector-target ratio.

Furthermore, involvement of NK activity was evaluated using YAC-1 target cells. Natural killer activity was detected 2 weeks postoperatively; however, effector cells amplified 4 weeks after grafting exhibited significant but low lytic activity of YAC-1 target cells (% specific lysis at an E/T ratio of 50/1 was 2.6% for KEP recipients and 0.3% for naive BALB/c; $n = 4$ for each group). The specific lysis of YAC-1 target cells was significantly suppressed by depletion of asialo GM1–positive cells (data not shown).

To further characterize the T-cell subset with allospecific killer activity, we next measured CTL activity after in vitro depletion of specific T-cell subsets. Splenocytes from grafted BALB/c mice, depleted of CD4$^+$ cells by in vitro incubation with anti-L3T4 antibody and rabbit complement, were assayed for allospecific killer activity. Although this treatment eliminated 92% of L3T4$^+$ cells, CTL activity to lyse RDM-4 target cells was not significantly affected (Fig. 3A). Next, splenocytes from grafted BALB/c mice, depleted of CD8$^+$ cells by incubation with anti-Lyt-2 antibody and complement, were also assayed for CTL activity. This treatment was shown to eliminate 96% of CD8$^+$ cells and was found to abolish allospecific CTL activity (Fig. 3B, $P < 0.05$). These findings indicate that CTL activity after KEP is mediated by the CD8$^+$ subset rather than the CD4$^+$ subset of T cells.
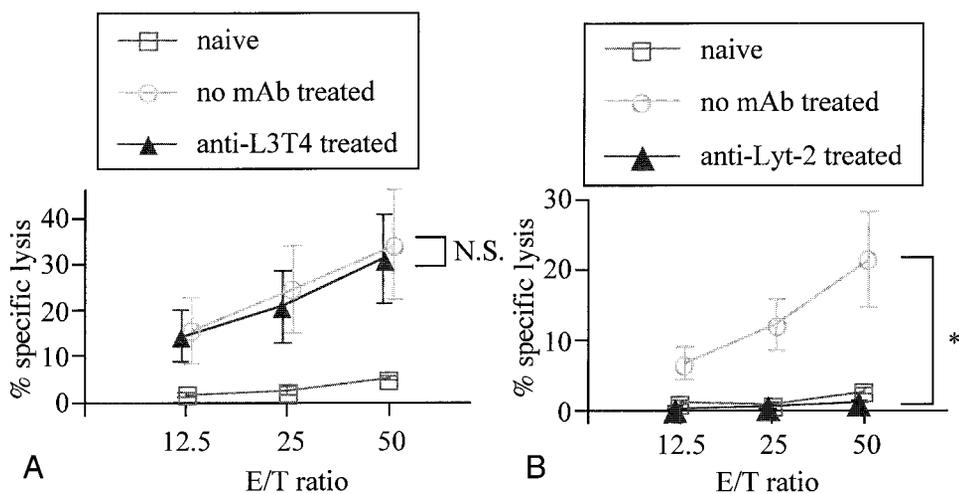
FIGURE 3. (A) Evaluation of CD4-mediated CTL activity after KEP. Recipient splenocytes were collected 3 weeks postoperatively, stimulated with irradiated C3H/He splenocytes, and incubated with anti-L3T4 antibody and rabbit complement. The CD4-depleted splenocyte effector cells were then assayed for allospecific CTL activity to lyse ^{51}Cr-labeled RDM-4 target cells (*$P < 0.05$, $n = 4$ for each group). (B) Allospecific CTL activity after in vitro depletion of CD8$^+$ cells 3 weeks postoperatively. Recipient splenocytes after in vitro incubation with anti-Lyt-2 antibody and rabbit complement were assayed for allospecific killer activity (*$P < 0.05$, $n = 4$ for each group). MAb, monoclonal antibody.
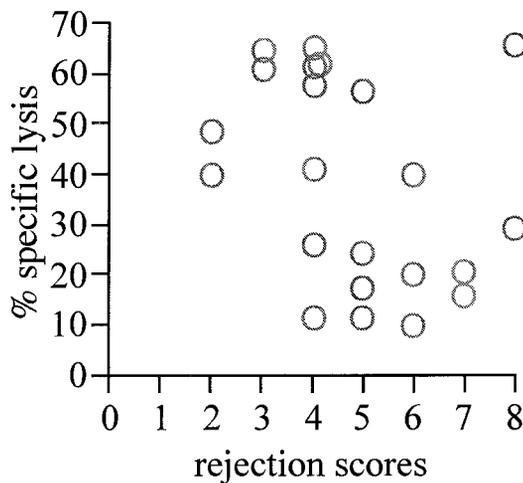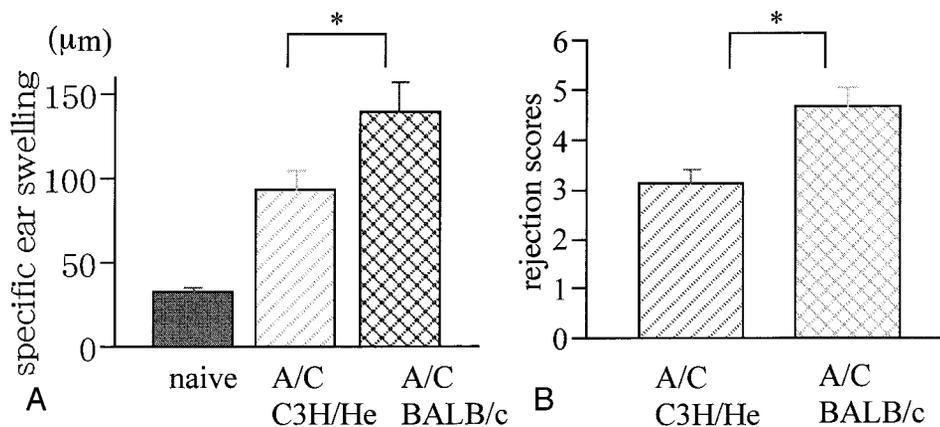
FIGURE 4. Reciprocality between allospecific CTL activity to C3H/He donor antigen and rejection scores at 2 weeks after KEP as assessed by Spearman correlation analysis ($r = -0.36$, $P = 0.076$, $n = 22$).

To determine whether this CTL activity actually participates in the rejection process, Spearman correlation analysis was performed to evaluate possible correlation of rejection scores to specific lysis of RDM-4 target cells 2 weeks postoperatively (Fig. 4). Unexpectedly, rather than a positive correlation, an inverse correlation was observed ($r = -0.36$, $P = 0.076$).

Suppression of Alloepithelial Rejection by Inhibition of DTH Response

We previously reported that suppression of the DTH response, induced by ACAID to minor antigens, correlates with suppression of corneal epithelial rejection.[11] To ascertain whether this phenomenon also takes place with both minor and major histocompatibility disparity, BALB/c recipients were grafted with C3H/He lenticules after ACAID induction. Figure 5A shows the DTH responses to C3H/He alloantigen of BALB/c recipients grafted with C3H/He lenticules 1 week after a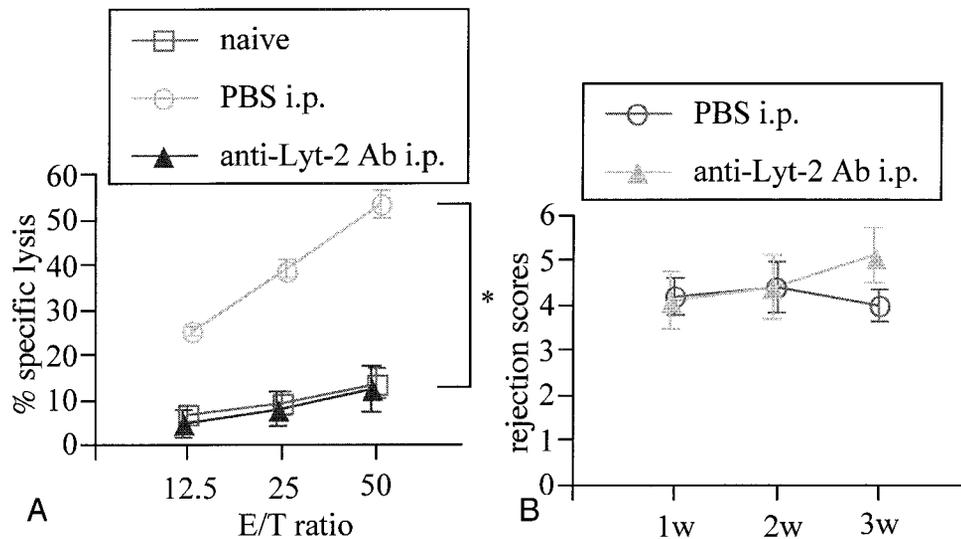nterior chamber inoculation of either C3H/He or BALB/c antigens. DTH responses against C3H/He donor antigen in recipients inoculated with C3H/He antigens were significantly lower than those inoculated with BALB/c antigens. Rejection scores of the grafted recipients are shown in Figure 5B. BALB/c recipients inoculated with C3H/He antigen had significantly decreased rejection scores when compared with control recipients inoculated with syngeneic antigen, although the rejection rates were 100% for both groups. The agreement between a downregulated DTH response and suppressed rejection provides further evidence that alloepithelial rejection is mediated mainly by DTH.

Effect of In Vivo Lyt-2+ Cells Depletion on Alloepithelial Rejection

To examine the role of CD8+ cells on the epithelial rejection in vivo, we treated recipients intraperitoneally with anti–Lyt-2 monoclonal antibody. Flow cytometric analysis of the spleen cells indicated that 96% depletion of Lyt-2+ cells was maintained throughout the observation period and that the L3T4+ population was not affected by the treatment. CTL activity to lyse RDM-4 target cells was measured on Lyt-2– depleted or PBS-treated recipients. Figure 6A shows that in vivo CD8+ cell depletion abolished the allospecific CTL response. However, the epithelial rejection rate of these recipients remained at 100% despite undetectable CTL activity, and rejection scores were comparable to control recipients 1 and 2 weeks postoperatively (Fig. 6B). Furthermore, 3 weeks postoperatively, the rejection scores increased over control, although this was not significant ($P = 0.11$). These results clearly show that alloepithelial rejection is not dominated by CTL responses.

Characterization of Infiltrating Cells Participating in Alloepithelial Rejection

Recipient eyes were enucleated at different phases of epithelial rejection for hematoxylin and eosin staining and immunohistochemistry. Reepithelialization of the denuded cornea was complete within a few days after grafting. Epithelial rejection was observed to start around day 7, with inflammatory cells

FIGURE 5. (A) Suppression of antigen-specific DTH due to anterior chamber (AC) inoculation of C3H/He splenocytes in BALB/c recipients. BALB/c recipients ($n = 11$ for each group) were inoculated in the anterior chamber with irradiated C3H/He splenocytes 7 days before KEP. Antigen-specific DTH against the donor antigen was measured at 2 weeks after KEP (*$P < 0.05$). (B) Suppression of rejection scores 2 weeks postoperatively by the induction of the anterior chamber–associated immune deviation. Anterior chamber inoculation of allogeneic C3H/He splenocytes significantly suppressed rejection when compared with inoculation with syngeneic antigens (*$P < 0.005$, $n = 28$ for each group).

FIGURE 6. (A) Abrogation of the allospecific CTL response by in vivo depletion of CD8^{+} T-cell subsets. Keratoepithelioplasty was performed in BALB/c mice that were depleted of CD8^{+} T cells by continuous administration of anti–Lyt-2 antibody (Ab) throughout the observation period. Recipient splenocytes were assayed for allospecific CTL activity to lyse ^{51}Cr-labeled RDM-4 target cells (*$P < 0.05$, $n = 4$ for each group). (B) Kinetics of rejection scores for CD8-depleted recipients. In vivo depletion of CD8^{+} T cells did not suppress allograft rejection ($n = 12$ for the PBS-treated control group, $n = 9$ in the anti–Lyt-2 antibody–treated group). i.p., intraperitoneal.

infiltrating areas around lenticules but few cells infiltrating the lenticular stroma initially. Subsequent corneal edema/opacity and neovascularization peaked at 2 weeks, with marked inflammatory cell infiltration then noted in lenticular stroma, subepithelially, and in the anterior stroma of the host cornea, and in the donor-derived epithelial layer on the host corneal bed. Throughout all phases, the inflammatory cells were predominantly composed of mononuclear cells and occasional neutrophils. Thy1.2 staining of these sections revealed that the mononuclear cells were composed mainly of T cells (Fig. 7A). Four days postoperatively, Thy1.2[+] cell infiltration was observed in the periphery of lenticules, but no positive staining for Thy1.2 was observed in the donor-derived corneal epithelium. There-

after, positive staining was observed to progress beyond the lenticules and reach the central host cornea at 1 to 2 weeks. This progression of Thy1.2 staining paralleled the development and progression of the epithelial rejection line observed by an operating microscope. The recruitment of Thy1.2^{+} cells peaked at 2 weeks, coinciding with the peaking of rejection scores. After the acute phase of rejection, Thy1.2^{+} infiltrating cells gradually decreased, with subsequent scarring and neovascularization observed.

To examine the involvement of T-cell subsets, L3T4 and Lyt-2 staining was performed. Both L3T4[+] and Lyt-2^{+} cells initially appeared in the periphery of lenticules, proceeding then to infiltrate the recipient's corneal bed by day 7 and
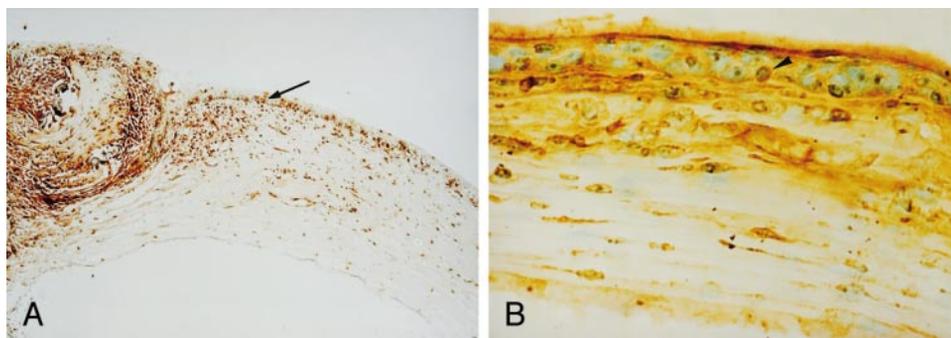
FIGURE 7. Immunohistochemical staining of grafted corneas 2 weeks after KEP. (A) Thy1.2[+] cell infiltration (*arrow*) into donor lenticules and donor-derived corneal epithelium. Enucleated eyes were fixed in PLP fixative and embedded in paraffin. Deparaffinized 6-µm–thick sections were processed for streptavidin–horseradish peroxidase staining. No background staining was observed using control IgG. (B) Interferon γ–positive cell infiltration (*arrowhead*) into the donor-derived epithelium on the host corneal bed. Frozen sections (7-µm–thick) were fixed in acetone and processed for streptavidin–horseradish peroxidase staining. Incubation with IgG was used for negative control staining. Specific positive cellular staining (*arrowhead*) was observed only in sections incubated with anti–interferon γ antibody.

peaking at 2 weeks. The infiltration of L3T4$^+$ cells was comparatively more intense than that of Lyt-2$^+$ cells.

Finally, we investigated whether infiltrating inflammatory cells in the KEP model produce either interferon γ, associated with the Th1 immune response, or IL-4, associated with the Th2 immune response. During the acute phase of rejection, interferon γ–positive cells were found to infiltrate the lenticules and corneal epithelium (Fig. 7B). To the contrary, no IL-4$^+$ cells were detected during the observation period.

DISCUSSION

Several researchers have reported that both DTH and CTL effector mechanisms contribute to the immunopathogenesis of allograft rejection in the PKP model.[6–10] However, in the KEP model, the roles of DTH and CTL responses in alloepithelial rejection had yet to be determined. To evaluate which cell-mediated immune mechanism dominates in allograft rejection after KEP, we used Spearman correlation analysis to directly assess correlation with clinical rejection scores. Preliminary analysis of correlation was performed using recipient mice at different times postoperatively (1–4 weeks) grouped together, and this showed significant positive correlation with respect to DTH, but not CTL activity (data not shown). Both DTH and CTL responses were detected by 2 weeks postoperatively and increased thereafter. However, because the onset of epithelial rejection occurred at 1 week, peaked at 2 weeks, and regressed thereafter, it may be inappropriate to group together clinical scores at different phases of rejection. Thus, we next analyzed correlation of clinical scores at one point (2 weeks postoperatively, Figs. 1B and 4) and found a clearly positive correlation with DTH, but not CTL activity. This result suggests that alloepithelial rejection after KEP is mediated mainly by DTH.

During the acute phase of the rejection, massive infiltration of T cells was observed in the lenticules and donor-derived epithelium (Fig. 7A). Both CD4$^+$ and CD8$^+$ cells were present, the former being relatively more abundant. Immunostaining of the alloepithelium revealed the infiltration of interferon γ–positive cells, indicating that Th-1 or Tc-1 cells had migrated into the donor-derived tissue and mediated allograft destruction by secreting proinflammatory cytokines. Because upregulation of DTH is mediated mainly by Th-1 cells, these findings support the hypothesis that DTH is responsible for rejection in our KEP model.

Using the combination of B10.D2 donors and BALB/c recipients, we previously determined that anterior chamber injection of donor specific lymphocytes reduces the incidence of alloepithelial rejection and suppresses allospecific DTH responses.[11] This combination, disparate only in multiple minor histocompatibility antigens, induces obvious alloepithelial rejection. In the present study, we used a donor and recipient combination disparate in both major and multiple minor histocompatibility antigens. Although the additional disparity of major histocompatibility antigens might induce distinct antigen processing, anterior chamber injection of donor antigens still induced a reduction in clinical scores and an allospecific DTH response. Because ACAID induces suppression of DTH with preservation of normal CTL and humoral antibody responses,[12] these results provide further evidence that alloepithelial rejection in KEP is mediated by DTH.

To examine the contribution of DTH to allograft rejection, we performed KEP after in vivo depletion of CD4$^+$ cells (data

not shown). Although some DTH suppression was achieved by depletion of CD4$^+$ cells, the allospecific DTH response could not be completely abolished. Because DTH developed even with 95% depletion of CD4$^+$ cells, we could not evaluate the contribution of DTH responses to allograft rejection using this approach. Indeed, DTH responses are reported to be capable of being mediated by CD8$^+$ and CD4$^+$ cells.[13] In the KEP model, CD8$^+$ cells may be inducing a DTH response, or enhancing a DTH response cooperatively with other effector cell subsets, when CD4$^+$ cell counts are low. To definitively demonstrate the contribution of DTH responses to allograft rejection using this approach, it would be necessary to use an experimental setting in which DTH responses are significantly inhibited or abolished.

In vitro depletion of CD8$^+$ cells demonstrated that the cytolytic activity of activated CTL precursors measured in our system was mediated by CD8$^+$ cells (Fig. 3), whereas cytolysis by CD4$^+$ T cells or NK cells was a minor event (Fig. 3A). Furthermore, in vivo depletion of CD8$^+$ cells abolished CTL responses (Fig. 6A), indicating that the induction phase of CTL activity is also mediated by CD8$^+$ cells. Abolishment of the CTL response did not suppress allograft rejection (Fig. 6B). These results further support the notion that the CD8$^+$ cell–mediated CTL response does not play an important role in allograft rejection.

Interestingly, suppression of the CTL response caused an increase in rejection scores after the acute phase of rejection (Fig. 6B), with rejection scores and CTL responses showing an inverse correlation (Fig. 4). This suggests that development of CTL responses might have an inhibitory effect on alloepithelial rejection. The animals that developed strong CTL responses may have concomitantly acquired suppressor CD8$^+$ subsets, which in turn regulated DTH responses by CD4$^+$ subsets. Depletion of CD8$^+$ cells might impair this suppressor activity, and thus contribute to increased rejection scores. Another possible explanation of this phenomenon may be the unique kinetics of the grafted epithelium. In the KEP model, the denuded host cornea is resurfaced by donor-derived epithelium within a few days after surgery; however, subsequent epithelial rejection results in destruction of the donor-derived epithelium and centripetal movement of host-derived epithelium. Consequently, after the onset of rejection, the host corneal bed is surfaced by a mosaic-like mixture of both recipient- and donor-derived epithelium (unpublished observations). When activated allospecific CTLs lyse donor-derived epithelium in the early phases of rejection, a decrease in alloantigen density may develop. Although this destruction of donor-derived epithelium by CTLs would itself constitute rejection, antigen-specific CTLs attack individual donor-derived epithelial cells in a pinpoint fashion as Ando et al.[14] demonstrated in the CTL-mediated liver disease model. In the PKP model, the attacked corneal endothelium suffers a devastating blow that leads to graft failure. In contrast, in KEP grafting CTLs will not affect the endothelium, only the corneal epithelium, the latter of which has a strong ability to proliferate. Thus, CTL-induced inflammation may not have a fatal impact on KEP grafting. The resultant decrease in antigen load, in turn, might alter or diminish enhancement of allospecific DTH. To the contrary, when a substantial amount of donor-derived epithelium remains as a result of insufficient CTL activity, the major histocompatibility complex–restricted stimulation of Th1 appears to elicit sufficient interferon γ release to induce a DTH response.[15] Moreover, Yamada and Streilein[16] suggest that the corneal epithelium on the graft itself promotes the development of DTH by secretion of inflammatory cytokines.

Taken together, these observations suggest that the CTL response does not play a major role in rejection in KEP, but rather that it modifies development of alloepithelial rejection.

In contrast to PKP, KEP is associated with relatively more frequent and more vigorous allograft rejection. In our previous report, using C57BL/6 donors and BALB/c recipients disparate in both major and minor histocompatibility antigens, rejection occurred in 100% of animals after KEP.[5] In a PKP model, C57BL/6 donors exhibited a 33% rejection rate.[17] When a high risk group of PKP recipients were grafted with C57BL/6 corneal buttons, the rejection rate increased to 100%, whereas induction of ACAID by intravenous injection of allogeneic peritoneal exudate cells cultured with transforming growth factor-β decreased the rejection rate to 37.5%.[18] In the present study, C3H/He donors with major and minor histocompatibility disparity to recipients also produced a 100% rate of rejection. However, this high incidence of rejection was not reduced by induction of ACAID, although clinical symptoms were significantly decreased. The difference in rejection rates between these PKP and KEP models cannot be explained by the intervention of ACAID alone.

In the KEP model, donor cornea with accompanying limbal tissue is secured along the limbus of the recipient cornea, such that recipient antigen-presenting cells have ready access to foreign antigens and can trigger immunologic sensitization. In addition, host-derived T cells, which are also in close proximity to the graft tissue, can infiltrate the graft and mediate allograft rejection. Donor-derived antigen-presenting cells might also migrate into the host tissue directly and initiate allograft rejection cooperatively, because the grafted lenticules include limbal as well as central areas of donor cornea. The direct pathway of alloantigen recognition might facilitate upregulation of the immunologic response in KEP. Involvement of direct alloantigen recognition is supported by our previous finding that both major and minor antigens are related to corneal epithelial rejection in KEP.[5] In a PKP model, Sano and colleagues[17] speculated that allorecognition by recipient T cells must occur by the indirect pathway of alloantigen processing because of the of the paucity of bone marrow–derived dendritic cells. In this situation, minor histocompatibility antigens may compete favorably with major histocompatibility complex antigens for presentation by recipient antigen-presenting cells.

In PKP, the CTL response is presumed to be more important than DTH because the main target of rejection is the endothelium, which has a slower rate of growth than the epithelium. Direct attack of individual cells by CTLs may thus be more fatal for the endothelium than for the epithelium. This notion is supported by data from Ksander and colleagues[19] who reported that donor-specific CTLs were not detected in draining lymph nodes of normal PKP recipients, but were detected in high-risk PKP recipients, and that their appearance coincided with graft rejection. In contrast, DTH responses in PKP are most likely weaker than those in KEP, because ACAID is induced by the PKP procedure itself.[6]

As was shown above, compared with the CTL response, the DTH response was more dominant in the immunopathogenesis of KEP. Although there are differences between KEP and PKP mechanisms that should be considered, the dominance of the DTH response over the CTL response in keratoplasty appears to also apply equally to PKP. He and associates[20] reported that in vivo CD4 depletion promotes graft survival but that CD8 depletion does not in the murine PKP model. Joo and colleagues[7] also reported that corneal allograft rejection after PKP of C57BL/6 donors to BALB/c mice is associated with the ability to generate strong DTH, and not CTL, responses. Thus, our KEP model is useful for increasing our understanding of the immunologic mechanisms of corneal transplantation as well as for analyzing the therapeutic effects and epithelial characteristics of clinical KEP in humans.

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