T Cell Subsets in the Immune Rejection of Murine Heterotopic Corneal Allografts

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Using a previously described murine heterotopic corneal allograft model, we examined the roles of delayed-type hypersensitivity (DTH) and cytotoxic T lymphocytes (CTL) in corneal allograft rejection. We have previously shown that normal C57BL/6 mice consistently reject heterotopic corneal allografts within 14 days of grafting. These hosts develop antigen-specific CTL responses but no evidence of DTH reactivity. The absence of DTH suggested that this T cell subset was unnecessary for corneal allograft rejection. The present studies using T cell-deficient mice selectively reconstituted with specific T cell subsets confirmed this suspicion. T cell-deficient (i.e., adult thymectomized, lethally irradiated, bone marrow-reconstituted = ATXBM) C57BL/6 mice were selectively reconstituted with the following categories of syngeneic lymph node cells (LNC): (1) BALB/c skin-immune LNC treated with anti-Lyt 1 antibody + complement; (2) BALB/c skin- or cornea-immune LNC treated with anti-Lyt 2 antibody + complement; or (3) BALB/c cornea- or skin-immune LNC not treated with antibody. ATXBM mice reconstituted with syngeneic Lyt 1-depleted, BALB/c skin-immunized LNC failed to develop DTH, yet rapidly rejected BALB/c corneal allografts. Similarly, ATXBM mice reconstituted with Lyt 1-depleted cornea-immune LNC did not demonstrate DTH responses but were able to reject 100% of the corneal allografts in an accelerated fashion. By contrast, corneal allograft rejection was significantly delayed in ATXBM mice reconstituted with cornea-immune LNC partially depleted of Lyt 2+ T cells. Collectively, the results indicate that: (1) heterotopic corneal allografts can be rejected in the absence of DTH; (2) heterotopic corneal allografts fail to induce allospecific DTH; and (3) partial depletion of Lyt 2+ CTL leads to a prolongation of heterotopic corneal allograft survival. Thus, the primary T cell-dependent immune effector elements responsible for heterotopic corneal allograft rejection appears to reside in the cytolytic T lymphocyte population. Invest Ophthalmol Vis Sci 27:1244-1254, 1986

Despite the high success rate of therapeutic keratoplasty, a significant number of human corneal allografts undergo immune rejection.1,2 Numerous studies have demonstrated the presence of Class I major histocompatibility complex (MHC) determinants on all corneal cellular elements,3-5 and recent reports indicate that the expression of class II MHC determinants on cultured rabbit6 and human7 corneal endothelial cells can be induced with gamma interferon. Thus, it is not surprising that donor-recipient matching for HLA loci correlates with improved corneal graft survival.8-10 Recent investigations have demonstrated that both fully allogeneic and MHC class I disparate heterotopic corneal allografts induce secondary cytotoxic T lymphocyte (CTL) responses11 as well as humoral antibody synthesis in rats.12 Similarly, we have recently reported that central corneal buttons grafted to heterotopic sites on fully allogeneic murine hosts induced primary CTL responses but, by contrast, delayed-type hypersensitivity responses failed to develop at any time after grafting.13 Thus, corneal allografts express MHC determinants that can induce at least some forms of allogimmunity and serve as targets for graft rejection.

The precise immune mechanisms responsible for corneal allograft rejection have yet to be defined; however, considerable evidence supports the view that immune rejection is T cell-mediated.14-16 Using a murine heterotopic corneal transplantation model,17 we examined the roles of delayed-type hypersensitivity (DTH) T cells and cytotoxic T lymphocytes in corneal allograft rejection. By placing corneal allografts onto a vascularized, non-privileged body site (i.e., subdermal graft bed), it was possible to examine corneal allograft rejection in an environment that favored the induction and expression of transplantation immunity. With this in mind, adult mice were rendered
T cell deficient followed by reconstitution with sensitized lymphoid cells that were selectively depleted of either Lyt-1⁺ (helper/inducer; DTH) or Lyt-2⁺ (CTL; suppressor) T cell subpopulations as a means of determining the phenotype of the T cells mediating corneal allograft rejection.

The results described in this report indicate that heterotopic corneal allograft rejection can occur in the absence of Lyt 1⁺, 2⁺ DTH T cells, and, by implication, can be mediated by Lyt 2⁺ CTL.

Materials and Methods

Experimental Animals

Adult female C57BL/6 (H-2b) and BALB/cJ (H-2*) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were used as experimental subjects when they were between 3-5 months of age. Surgical procedures were performed on mice anesthetized with ether or sodium pentobarbital. The present investigations conform to the ARVO Resolution on the Use of Animals in Research.

Production of T Cell-Deficient Mice (ATXBM)

Adult C57BL/6 mice were anesthetized by intramuscular injection of sodium pentobarbital (1-2 mg/mouse) and thymectomized as previously described.18 Thymectomized mice were placed on acidified drinking water (pH 2.8) followed 2 weeks later by lethal whole body irradiation (850 rad, 137Cesium source). Mice were rescued by intravenous reconstitution with 5 X 10⁶ syngeneic bone marrow cells (BM) depleted of T cells by treatment with monoclonal antibody anti-Thy 1.2 and complement as described below.

Production of ATXBM-Thy 1.2⁻, ATXBM-Lyt 1.2⁻ and ATXBM-Lyt 2.2⁻ Mice

Lymph node cells (LNC) were harvested from normal C57BL/6 mice or from C57BL/6 mice sensitized 2 weeks prior by grafting BALB/c skin or cornea onto the chest wall as described below. 5 X 10⁷ normal or sensitized LNC were adoptively transferred to ATXBM mice by intravenous injection. Prior to adoptive transfer, LNC were either untreated or depleted of Thy 1.2⁺, Lyt 1.2⁺, or Lyt 2.2⁺ T cells by two cycles of lysis with monoclonal antibodies and complement as described below. All experimental mice were allowed a 2-3 week recovery period before cornea or skin grafting.

Monoclonal Antibodies (Mab)

Anti-Thy 1.2 Mab (Cedarlane Laboratories; Ontario, Canada) and Anti-Lyt 1.2 Mab (New England Nuclear; Boston, Mass.) were IgG2B isotypes. Mab Anti-Lyt 2.2 (New England Nuclear) was an IgM isotype. All Mabs were supplied in ascites fluid and were shown by Chromium-release assay to be directly cytotoxic to C57BL/6 LNC in the presence of rabbit complement. The percentage of cells depleted from LNC suspensions was well within the expected range for Thy 1⁺, Lyt 1⁺, and Lyt 1⁺Lyt 2⁺ T cells within lymph nodes. All Mabs were used at a 1:1000 dilution for depletion experiments.

T Cell Depletions

T cells or T cell subsets were depleted from LNC or BM suspensions by incubation with Mabs Thy 1.2, Lyt 1.2, or Lyt 2.2 plus complement. LNC or BM were incubated [5 X 10⁷ cells/ml in HBSS, 0.3% bovine serum albumin (BSA)] with Mabs for 45 min on ice. The cells were washed (200 X G 10 min), resuspended (5 X 10⁷ cells/ml in RPMI, 0.3% BSA) and T cells were lysed by incubation with rabbit complement (RC; 1:10 dilution; Low-Tox-M, Cedarlane Laboratories, Ontario, Canada) for 45 min at 37°C in a 5.0% CO₂ atmosphere. Depletion of T cells or subsets was confirmed by functional assays as described below.

Skin Grafting

Full-thickness grafts (25 mm²) fashioned from abdominal skin were prepared from BALB/c donors and grafted orthotopically onto the thoracic wall of C57BL/6 recipients as described elsewhere.19 Grafting procedures were carried out with recipient mice placed under ether anesthesia. Mice with viable, intact grafts on day 7 post-transplantation were used as LNC donors in subsequent experiments.

Cornea Grafting

Full-thickness central corneal C57BL/6 syngrafts or BALB/c allografts were prepared and grafted heterotopically onto a subdermal graft bed as described previously.17 Care was taken to cut away all limbal tissue leaving a corneal graft approximately 2 mm in diameter.13 Two corneas were grafted to each experimental and control animal. Plaster casts were removed 7 days after grafting and corneal grafts were observed for evidence of rejection by slit-lamp biomicroscopy. After observation and graft scoring, new dressings and plaster of Paris bandages were applied. Grafts were observed and scored 7, 14, 21, and 28 days post-transplantation.

Cornea Graft Observation and Scoring

The following observations were made with the aid of a slit-lamp microscope by a single experienced observer: loss of clarity; degree of convex contour, as a measure of edema; neovascularization originating in...
the peripheral or posterior aspect of the corneal graft; vessel activity; vessel density; the degree of cellular infiltrate; and the degree of dryness and keratinization. A score was given for each category based on an arbitrary scale of 0 to 4+. An index of rejection was calculated based on the average combined score for loss of graft clarity and the degree of cellular infiltrate for all mice in a panel at a given time after grafting. In our experience, these parameters were the best indicators of graft prognosis. The percentage graft survival recorded on each observation day was based on the number of viable grafts remaining on post-operative day 7. Grafts lost before day 7 were scored as technical failures.

Cell-Mediated Cytotoxicity Assays

Spleens and lymph nodes from grafted and naive control C57BL/6 mice were removed aseptically. Single-cell suspensions were prepared by gently teasing the organs in cold Hanks balanced salt solution (HBSS). Erythrocytes in spleen cell suspensions were lysed by Tris-NH4Cl. Spleen and lymph node cell (LNC) suspensions were washed (200 × G, 10 min) and resuspended in complete medium [RPMI 1640 (KC Biological; Lenexa, Kansas) containing heat-inactivated 10% fetal bovine serum (Hyclone; Logan, Utah), 2.0 mM L-glutamine (Gibco; Grand Island, NY), 5 × 10^{-5} M β-mercaptoethanol, 10 mM Hepes buffer, 100 IU penicillin/ml, 0.25 μg Fungizone and 100 μg of streptomycin/ml] at a concentration of 5 × 10^6 cells/ml.

BALB/c and B10.BR spleen stimulator cells were prepared as described and irradiated (3000 R; 137Cesium source). Responder and stimulator cells (5 × 10^5 of each population in a total volume of 2.0 ml) were added to 24 well trays (Costar Plastics) and incubated at 37°C in a humidified 5% CO2 atmosphere. Effector cells were harvested from boost cultures after 36–48 hr. Cytotoxicity was determined in a 4-hr 51Chromium-release assay as described elsewhere.20 P815 mastocytoma (H-2b, DBA/2 origin) or B10.BR (H-2k) Con-A stimulated blasts were labeled by incubation with Na2^{51}CrO4 (New England Nuclear, Boston, MA) at 200 μCi/ml/10^7 cells in complete medium at 37°C for 45 min and served as target cells. Blast target cells were prepared by culturing B10.BR spleen cells (1 × 10^6 cells/ml) for 3 days in complete medium supplemented with 2.0 μg/ml concanavalin A (Sigma Chemical Co.; St. Louis, MO) and 15% supernatant derived from Con-A activated (5.0 μg/ml) rat spleen cells. Effector and target cells were cultured in round-bottom microtiter plates (Falcon; Oxnard, CA) in a total volume of 200 μl with effector:target cell ratios ranging from 20:1 to 2.5:1. Plates were centrifuged at 100 × G for 2 min followed by 4-hr incubation at 37°C in a humidified 5% CO2 atmosphere. Following incubation, plates were centrifuged for 5 min at 200 × G and 100 μl of supernatant from each well was removed and counts per min (cpm) were determined in a gamma counter (Tracor Analytic). Cytotoxicity of each effector population was determined by the following formula:

\[
\text{% Specific }^{51}\text{Cr-release} = \frac{[\text{experimental cpm} - \text{spontaneous release cpm}]}{\text{maximum release cpm}} \times 100 \pm \text{SE}.
\]

Maximum 51Cr-release was determined by detergent lysis of target cells and spontaneous 51Cr release was determined by culturing target cells in medium only.

Delayed-Type Hypersensitivity (DTH) Assay

DTH responses to allografts were measured by a conventional footpad swelling assay as described elsewhere.21 Panels consisted of experimental C57BL/6 mice (Table 1), normal age-matched negative controls, and C57BL/6 mice grafted 2 weeks prior with BALB/c skin grafts as positive controls. Footpads were challenged and measured for DTH responses 14 days after grafting. Both hind footpads of each mouse were measured with an engineer’s micrometer (Mitutoyo Corp.; Tokyo, Japan) immediately before footpad challenge. An eliciting dose of 1 × 10^7 γ-irradiated (3000 R) BALB/c LNC suspended in 25 μl of HBSS was injected into the subcutaneous tissue of the right hind footpad. The left hind footpad served as a negative control and received 25 μl of HBSS without LNC. Both footpads were measured 24 and 48 hr later and the difference in footpad thickness was used as a measure of DTH. Results are expressed as footpad swelling where:

\[
\text{footpad swelling} = \frac{[(24 \text{ hr} - 0 \text{ hr measurement, experimental foot}) - (24 \text{ hr} - 0 \text{ hr measurement, control foot})] \times 10^{-3} \text{ mm} \pm \text{SE}}.
\]

Statistical Analysis

Statistical significance was determined by the Student’s T-test for the difference between two independent means.

Results

The experiments described below were designed to determine the phenotype of the T cells that mediate corneal allograft rejection. Adult C57BL/6 mice served as hosts and were rendered T cell-deficient by thymectomy, lethal whole-body γ-irradiation (850 R), and reconstitution with syngeneic bone marrow depleted of T cells by treatment with anti-Thy 1.2 plus complement. Panels of adult thymectomized bone marrow-
Table 1. Description and treatment of mouse panels

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<th>Panel designation</th>
<th>Thymectomized, irradiated, BM-reconstituted</th>
<th>Source</th>
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<th>Skin/cornea grafted</th>
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reconstituted (ATXBM) mice were restored with syngeneic lymph node cells (LNC) selectively depleted of specific T cell populations. The various categories of selectively immune reconstituted mice are summarized in Table 1.

Corneal Allograft Rejection by Normal Mice and T Cell-Reconstituted ATXBM Mice

As reported in previous studies, C57BL/6 corneas grafted to a heterotopic site on syngeneic recipients survive for prolonged periods (>21 days). Despite an initial increase in rejection index (RI) and a corresponding decrease in graft survival during the first 10-14 days following transplantation of the corneal syngrafts, these values begin to level or decline by 21 days post-transplantation (Fig. 1). By contrast, BALB/c heterotopic corneal allografts placed onto T cell-deficient ATXBM C57BL/6 mice showed minimal loss of clarity and no infiltrate 7 days after grafting as indicated by the low RI value (0.4-0.65) observed on this day (Fig. 2A). On day 14, a slight increase in RI and a decreased survival value (Fig. 2B) was noted but there was no progression in the inflammatory response and by day 21, the infiltrate had decreased markedly. The observed mild inflammatory reaction was attributed to a nonspecific response to the surgical procedure. At day 14, 70-75% of the corneal grafts survived and were easily identifiable as corneal tissue (Fig. 2B). The number of surviving corneal grafts remained stable through day 21 and by 28-30 days post-transplantation, 50% of the corneal allografts were still intact. Thus, BALB/c corneal allografts escaped rejection in the T cell-deficient allogeneic hosts.

By contrast, C57BL/6 ATXBM mice reconstituted with normal syngeneic LNC rejected their corneal allografts (Fig. 2). On day 7 post-transplantation, the values recorded for graft clarity and cellular infiltrates were not significantly different in magnitude from those parameters in ATXBM T cell-deficient mice. However, by day 14 there was an increase in the inflammatory response which steadily progressed during the following 2 weeks and peaked at day 28 (RI = 2.70-3.1). By day 28, the majority of the corneal allografts had been destroyed by the inflammatory process and only 10-15% of the grafts survived as identifiable corneal tissue (Fig. 2B).

ATXBM mice reconstituted with immune LNC from donor mice sensitized with BALB/c skin grafts showed an accelerated rejection response (Fig. 2).
was evidence of an early inflammatory response by day 7, with the greatest intensity occurring by day 14. On day 14, only 23% of the corneas survived and by day 21 no identifiable corneas remained.

Thus, T cell-deficient ATXBM mice fail to reject corneal allotrafts. However, the ability of T cell-deficient mice to reject corneal allografts can be restored by the adoptive transfer of either normal or pre-immune lymphoid cells.

**Corneal Allograft Rejection by Lyt 1-Depleted ATXBM Mice**

In the next series of experiments we wished to determine the phenotype of the lymphoid cells mediating corneal allograft rejection. Accordingly, panels of ATXBM mice were reconstituted with immune lymphoid cells harvested from syngeneic donor mice that had recently rejected BALB/c skin allografts. Lymphoid cells were depleted of Lyt 1+ cells by in vitro treatment with monoclonal anti-Lyt 1.2 antibody in the presence of complement. This treatment resulted in the lysis of approximately 65% of the lymphoid cells used for adoptive transfer and was within the range of the Lyt 1+ LNC reported by others. 22-23

The results in Figure 3 indicate that C57BL/6 ATXBM mice reconstituted with Lyt 1-depleted immune LNC rapidly rejected their corneal allografts. The grafts showed evidence of rejection by day seven with loss of clarity and the presence of infiltrates (RI = 1.75). By day 14, both parameters had progressed and only 15% of the corneas survived. The pattern of rejection in Lyt 1-depleted ATXBM mice was similar to that seen in ATXBM mice reconstituted with untreated immune LNC. Thus, removal of Lyt 1+ lymphoid cells does not impair corneal allograft rejection and suggests that DTH is not a crucial component of this process.

The effectiveness of the Lyt 1-depletion was confirmed by functional assays of DTH responsiveness in the grafted mice. Panels of ATXBM mice that were reconstituted with either untreated immune or Lyt 1-depleted immune LNC were tested for DTH activity by a conventional footpad swelling assay 2 weeks after receiving corneal grafts. Positive controls consisted of normal C57BL/6 mice grafted with BALB/c skin 2 weeks prior to the footpad swelling assay. Ungrafted normal and grafted ATXBM C57BL/6 mice served as negative controls. The results indicate that skin grafted C57BL/6 mice as well as ATXBM mice reconstituted with immune LNC mounted vigorous DTH responses to BALB/c alloantigens (Fig. 4). By contrast, ATXBM mice reconstituted with Lyt 1-depleted immune LNC failed to develop significant (P > 0.05) DTH reactivity, even though they had previously rejected corneal allografts.

Thus, the immune rejection of corneal allografts can occur in the absence of DTH and is not mediated by Lyt 1+ T cells.
Corneal Allograft Rejection by Lyt 2-Depleted ATXBM Mice

The finding that Lyt 1-depleted ATXBM mice were able to reject corneal allografts while their ATXBM T cell-deficient counterparts failed to reject similar grafts implies that Lyt 2^+^ T-cells may be the primary mediators of corneal graft destruction. Additional experiments tested this hypothesis.

Panels of ATXBM mice were reconstituted with either untreated or anti-Lyt 2-treated immune LNC prior to receiving heterotopic corneal allografts. Surprisingly, ATXBM mice reconstituted with anti-Lyt 2-treated immune LNC were able to reject their corneal allografts at a rate comparable to that found in ATXBM mice reconstituted with untreated immune LNC (Figure 3). At day 14, 20% of the grafts survived and by day 28 no corneal tissue could be identified in the graft beds.
Skin Graft

Immune

ATXBM

Immune LNC

ATXBM +

Lyt 1− LNC

ATXBM +

Lyt 2− LNC

0 40 120 240 360 480

Specific Footpad Swelling (X 10^−3 mm)

Fig. 4. DTH responses in cornea-grafted ATXBM mice reconstituted with LNC selectively depleted of specific T cell subsets. C57BL/6 mice (six mice per panel) received either two heterotopically placed BALB/c corneal allografts or one BALB/c orthotopic skin allograft. DTH was measured by footpad swelling 14 days after grafting. Normal age-matched mice served as negative controls. DTH is expressed as specific footpad swelling (X 10^−3 mm) 24 hours after footpad challenge with 1 X 10^7 7-irradiated (3000 R) BALB/c LNC. Bar represents ±S.E. * = Significantly different (P < 0.01) from negative control.

Although treatment of the LNC suspensions with anti-Lyt 2.2 monoclonal antibody removed 20% of the lymphoid cells and was within the expected range for the number of Lyt 2+ cells within lymph nodes, it is possible that a small, albeit significant, number of CTL precursors escaped antibody destruction and proliferated following adoptive transfer to the ATXBM recipients. Additional functional assays were performed to test this hypothesis. The various panels of experimental mice that had rejected their corneal allografts were killed and their lymph node cells examined for antigen-specific CTL activity in a standard cell-mediated cytotoxicity assay. LNC harvested from ATXBM Lyt 1-deficient mice displayed cytotoxicity toward P815 target cells expressing the H-2 alloantigens of BALB/c (Fig. 5A). By contrast, T cell-deficient ATXBM mice failed to generate CTL responsiveness to BALB/c alloantigens even after in vitro restimulation with BALB/c spleen cells (Fig. 5B). To our surprise, however, significant (P < 0.01) CTL activity was also detected in the ATXBM mice reconstituted with anti-Lyt 2-treated LNC. Thus, a single in vitro treatment with anti-Lyt 2 plus complement was insufficient for removing CTL from the adoptive transfer cell suspensions.

Additional experiments employed a more aggressive depletion procedure in which immune LNC suspensions were subjected to two sequential treatment cycles

Fig. 5. CTL responses in cornea-grafted mice. C57BL/6 mice received two BALB/c corneal allografts on the lateral thorax. Experimental ATXBM C57BL/6 mice (A) were reconstituted with anti-Lyt 1.2-treated LNC (○) or anti-Lyt 2.2-treated LNC (●) harvested from C57BL/6 mice that had been grafted 14 days prior with BALB/c skin allografts. Control groups (B) consisted of: naive C57BL/6 mice (●); normal C57BL/6 mice grafted 14 days prior with allogeneic skin (○); central corneal buttons (●) from BALB/c donors; T cell-deficient ATXBM mice (△); and ATXBM mice fully reconstituted with skin-immune LNC (λ). LNC were harvested from naive and grafted animals 14 days post-transplantation and tested for CTL responses against 51chromium-labeled P815 (H-2b) target cells after culturing 5 X 10^6 effector cells for 36 hr with 5 X 10^6 7-irradiated BALB/c spleen cells. Lysis of third party B10.BR (H-2d) blast targets was always less than 5% specific 51chromium-release. Bar represents ±S.E. for a panel of three mice.
Corneal allograft rejection by ATXBM mice reconstituted with cornea-immune LNC. C57BL/6 mice received two BALB/c heterotopic corneal allografts on day 0. ATXBM mice (10-20 mice per panel) were fully reconstituted with immune LNC from C57BL/6 mice that had been grafted 14 days prior with BALB/c cornea (●) or BALB/c skin (▲) allografts. Additional ATXBM mice were reconstituted with cornea-immune LNC after treatment of LNC with anti-Lyt 2.2 monoclonal antibody and complement (○). Average rejection index (A) and percentage graft survival (B) were calculated for each panel of mice on a given day as described in Figure 1. Bar represents ±S.E.

with anti-Lyt 2 antibody in the presence of complement. ATXBM mice reconstituted with these LNC showed a modest reduction in the inflammatory reaction on days 7 and 14 compared to their counterparts reconstituted with immune LNC treated with a single cycle of anti-Lyt 2 antibody (data not shown). It was noted that 63% of the corneal grafts survived at day 14 and 11% survived at day 21. In those corneas that survived past day 14, there was a slight reduction in the inflammatory reaction; however, by day 28 100% of the corneas had been rejected by their hosts. Moreover, ATXBM mice reconstituted with untreated cornea-immune LNC suspensions treated with 2 cycles of anti-Lyt 2 antibody developed antigen-specific CTL (data not shown). This finding did not come as a surprise since treatment of immune LNC suspensions with two cycles of anti-Lyt 2 antibody did not remove substantially more T cells than a single treatment with this same antibody (i.e., 20% depletion of total lymphocytes). Thus, we were not able to deplete the CTL functional activity in experimental mice while leaving their DTH responsiveness intact.

Corneal Allograft Rejection by ATXBM Mice Reconstituted With Cornea-Immune LNC

We have recently reported that central corneal allografts from BALB/c donors induce a primary CTL response in C57BL/6 recipients, yet no DTH response to corneal alloantigens can be detected at any time after grafting. Since corneal allografts immunize the host for CTL but not DTH activity, we tested the ability of untreated and Lyt 2-depleted LNC from cornea-immunized donors to transfer immunity to ATXBM recipients. Accordingly, ATXBM mice were reconstituted with LNC from syngeneic mice that had previously rejected BALB/c corneal allografts and were tested for their ability to reject corneal allografts in an accelerated fashion. ATXBM mice reconstituted with untreated cornea-immune LNC rejected subsequent corneal allografts at a tempo identical to ATXBM mice reconstituted with skin-immune LNC (Fig. 6). By contrast, ATXBM mice reconstituted with Lyt 2-depleted cornea-immune LNC demonstrated a reduced capacity to reject their corneal allografts and 7% of the grafted corneas (two recipients) survived beyond 30 days. Although only a modest effect was obtained, these data provide further evidence that CTL play an active role in the rejection of corneal allografts from heterotopic sites.

Discussion

Immune rejection is the leading cause of corneal allograft failure. In spite of numerous animal and human studies, the precise immune mechanisms leading to corneal allograft destruction remain a mystery. Animal studies have shown that corneal graft rejection is a cell-mediated phenomenon; however, the identity of the effector cells has yet to be characterized.

Transplantation biologists have relied on two basic approaches for studying the mechanisms of allograft rejection. The first method is to determine, by immunohistology, the surface phenotypes of immune cells infiltrating rejecting allografts. A second approach is to determine whether T cell-deprived ATXBM rats and
mice are able to reject organ allografts following selective reconstitution with specific T cell populations (i.e., T helper cells or cytotoxic T cells). Both experimental approaches have distinct advantages and disadvantages. Moreover, investigators employing these two methodologies have come to diametrically different conclusions regarding the importance of CTL and DTH in the rejection of allografts. Numerous studies, utilizing the adoptive transfer of specifically sensitized T cell populations, have supported the notion that CTL are the dominant immune effector for various categories of allografts, while other investigations report that allograft rejection is strictly dependent upon DTH mediated processes. The controversy over which T cell population is pivotal in allograft rejection is further complicated by studies on the rejection of syngeneic male skin grafts by female recipients. The immune response gene control of DTH reactivity directed against H-Y (male) antigens appears to be indistinguishable from that governing skin graft rejection. By contrast, Lubaroff reported that sublethal, whole-body irradiation eliminates DTH responsiveness, yet fails to prevent skin allograft rejection in the rat. Thus, cogent arguments can be promulgated to both support and refute the importance of either CTL or DTH-dependent immune mechanisms of graft rejection. Nonetheless, the weight of evidence supports the view that the T cell is the key immune effector element in the rejection of corneal allografts as well as other solid tissue grafts.

Recent immunohistologic investigations of human corneal allografts undergoing rejection demonstrated a mixed inflammatory infiltrate comprised of both T helper (Leu 3a) and cytotoxic/suppressor T cells (Leu 2a) cells, with a slightly higher number of the T helper cell population. Similar studies examining paralimbal biopsy specimens taken from patients at the time of corneal allograft rejection have shown the presence of stromal and epithelial infiltrates comprised chiefly of the cytotoxic/suppressor T cell subset. Although such studies provide important clues to the possible mechanism of allograft rejection, they should be viewed with caution. We agree with the statements of Pepose et al that in situ studies of the cellular infiltrates present in biopsy specimens of rejecting allografts are difficult to interpret since the immunohistological identification of a particular cell subset does not necessarily confirm that cell’s primary participation in allograft rejection. For example, it is possible that many of the inflammatory cells found in rejecting allografts appear in response to tissue damage mediated by other populations of immune cells that are the primary effectors of graft damage.

T cell-deprived ATXBM rats and mice have been used extensively for examining the roles of specific T cell subsets in skin allograft rejection. However, the major disadvantage of this approach is the extreme difficulty in producing a truly CTL-deficient animal.

In the present study we employed the ATXBM mouse as a tool for determining the roles of CTL and DTH in the rejection of heterotopic corneal allografts. Although we are aware of the possible disadvantages of this animal model, we believe that it offers significant advantages over the other experimental approaches described above. For example, it is possible to produce a DTH-deficient ATXBM mouse and thereby examine the role of CTL in an otherwise T cell-deficient mouse. Moreover, we believe that DTH functional deficiency is a valid indication of the total elimination of the effector T cells mediating DTH responses (i.e., Lyt 1-2 T cells), since Marchal et al demonstrated, by limiting dilution analyses, that it was possible to detect a footpad swelling response in mice after adoptive transfer of a single sensitized antigen-reactive T cell.

Using this approach, we examined the role of Lyt 1+ (DTH/helper) and Lyt 2+ (cytotoxic/suppressor) T cell populations in the rejection of corneal allografts. The results provide a number of insights into the immunobiology of corneal allograft rejection. As in previous studies, we found that corneal allograft rejection was a T cell-dependent process. However, experiments using Lyt 1-deficient mice indicated that this T cell subset was not necessary for corneal allograft rejection. Hosts reconstituted with Lyt 1-depleted LNC suspensions were able to reject their grafts, yet failed to mount antigen-specific DTH responses directed against the corneal alloantigens. Thus, corneal allografts can be rejected without the participation of a DTH effector component. These findings do not rule out the possibility that DTH may, under certain conditions, participate in corneal graft rejection; however, we have recently reported that central corneal allografts are unable to induce DTH responsiveness in normal immunocompetent hosts even though such grafts are rejected swiftly. The apparent absence of antigen-specific DTH reactivity before, during, and after central corneal allograft rejection is particularly noteworthy since the footpad assay used in these studies is an exquisitely sensitive method for detecting delayed-type hypersensitivity in the mouse. Thus, the inability of central corneal allografts to evoke DTH reactivity, combined with the present findings that Lyt 1+ effector cells are unnecessary for the immune rejection of corneal allografts, make strong arguments against a role for DTH as a leading cause of heterotopic corneal graft destruction.

The rejection of corneal grafts in the absence of systemic DTH reactivity implies that CTL are the primary effector cells in this process. This hypothesis would be
best proven by the longterm survival of corneal allografts on CTL-deficient mice. However, our efforts to create mice functionally depleted of CTL activity were unsuccessful even when the ATXBM hosts were reconstituted with skin-immune LNC suspensions treated aggressively by two sequential cycles with anti-Lyt 2 antibody plus complement. In spite of these exhaustive procedures, these ATXBM hosts rejected their corneal allografts and developed antigen-specific CTL responses. By contrast, the prolonged survival and reduced rejection index of corneal allografts from several ATXBM mice reconstituted with Lyt 2-depleted cornea-immune LNC suggest that CTL play a dominant role in the rejection of heterotopic corneal allografts. Our inability to create CTL deficient ATXBM mice is not unexpected, since LeFrancois and Bevan reported that ATXBM mice reconstituted with anti-Lyt 2-treated LNC suspensions developed CTL. These investigators determined that the CTL found in such hosts were derived from radiosensitive CTL precursors present in the recipient's bone marrow. Thus, it is extremely difficult to render mice devoid of CTL.

The present findings suggest that corneal allograft rejection can occur in the absence of DTH and, thus, implies a dominant role for antigen-specific CTL. However, this conclusion may be overly simplistic and not applicable for all cases of corneal allograft rejection. We have reasoned that the failure of central allografts to induce systemic DTH responsiveness rules out this immune process as a cause for graft destruction. However, we have recently found that full-diameter corneal allografts that contain limbal Langerhans cells are capable of eliciting vigorous systemic DTH reactivity (unpublished findings). Therefore, corneal grafts that contain resident donor-derived Langerhans cells are potent inducers of DTH and are potentially vulnerable to rejection by this cellular immune mechanism. One can only speculate as to whether corneal allograft rejection in humans is either initiated or exacerbated by grafts on CTL-deficient mice. However, our efforts to create mice functionally depleted of CTL activity were unsuccessful even when the ATXBM hosts were reconstituted with skin-immune LNC suspensions treated aggressively by two sequential cycles with anti-Lyt 2 antibody plus complement. In spite of these exhaustive procedures, these ATXBM hosts rejected their corneal allografts and developed antigen-specific CTL responses. By contrast, the prolonged survival and reduced rejection index of corneal allografts from several ATXBM mice reconstituted with Lyt 2-depleted cornea-immune LNC suggest that CTL play a dominant role in the rejection of heterotopic corneal allografts.

Although CTL-mediated corneal graft rejection has not been proven, it is strongly implicated.

**Key words:** corneal allograft rejection, T cells, delayed-type hypersensitivity, cytotoxic T cells

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