Promotion of Murine Orthotopic Corneal Allograft Survival by Systemic Administration of Anti-CD4 Monoclonal Antibody

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A mouse model of orthotopic corneal allograft rejection was used to examine the efficacy of anti-CD4 and anti-CD8 monoclonal antibodies in preventing immunologic rejection of corneal allografts. Although it is believed by many that corneal graft rejection is mediated, at least in part, by CD8-positive cytotoxic T-lymphocytes, systemic administration of anti-CD8 antibody did not reduce the rejection rate of corneal allografts that differed from the host at the entire major histocompatibility complex. By contrast, systemic administration of anti-CD4 monoclonal antibody reduced the rejection rate from 83% (untreated controls) to 33%. Fluorocytometric analysis of residual lymphoid populations showed that neither monoclonal antibody eliminated the inappropriate subset of T-cells in antibody-treated animals. In vitro cell-mediated cytotoxicity assays showed that both antibodies eliminated allospecific cytotoxic T-lymphocyte populations; however, only anti-CD4 antibody promoted graft survival. Thus, these results indicate that anti-CD4 monoclonal antibody is a powerful immunosuppressive agent for promoting corneal graft survival and that CD8-positive T-cells alone do not cause rejection of corneal allografts. Invest Ophthalmol Vis Sci 32:2723-2728, 1991

Recent advances in developing T-cell subset-specific monoclonal antibodies (mAbs) offer promising alternatives for suppressing the allograft immune response. Such mAbs may be useful because they target specific T-cell populations without producing the side effects described for the immunosuppressants. Several laboratories reported that systemic treatment with either anti-CD4 or anti-CD8 mAbs resulted in prolonged graft survival in skin, heart, and kidney allograft models.4 We evaluated the effect of systemic treatment with anti-CD4 and anti-CD8 mAbs on the survival of corneal allografts that were mismatches at the entire major histocompatibility complex (MHC) and multiple minor H loci. These results showed that, although anti-CD8 mAb did not promote corneal graft survival, anti-CD4 mAb treatment greatly reduced the rejection of corneal allografts.

Materials and Methods

Mice

Female BALB/c (H-2b) and C57BL/6 (H-2b) were obtained from Jackson Laboratories (Bar Harbor, ME); they were 2–6 months old. All experimental animals were handled in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and the ARVO Resolution on the Use of Animals in Research.
Monoclonal Antibodies (mAb)

The GK 1.5 hybridoma (American Type Culture Collection, Rockville, MD) secretes a rat anti-mouse CD4-specific antibody of the immunoglobulin G2b isotype.\(^5\) The YTS 169.4 hybridoma (a gift from Dr. M. Bennett, University of Texas Southwestern Medical Center, Dallas, TX) secretes a rat anti-mouse CD8-specific antibody of the immunoglobulin G2b isotype.\(^5\) Both hybridomas were propagated in nude mice and the monoclonal antibodies isolated from ascites fluid. Antibodies were affinity purified over protein A columns and MAPS II monoclonal antibody purification system (BIO-RAD, Richmond, CA). Purified antibody was concentrated using an Amicon 8200 ultrafiltration cell with molecular cutoff of 10,000 (WR Grace, Danvers, MA). Protein concentrations were determined using bicinchoninic acid (BCA) reagents (Pierce, Rockford, IL) and a DU 64 spectrophotometer (Beckman, Fullerton, CA). Final protein concentration was adjusted to 0.5 mg/ml. The secondary antibody for use in fluorocytometry was fluorescein isothiocyanate (FITC)-labeled mouse anti-rat immunoglobulin G (Jackson ImmunoResearch, West Grove, PA).

Because thymectomy was shown to improve the T-cell depletion produced by anti-CD4 and anti-CD8 antibodies,\(^4\) all mice were thymectomized 2 weeks before mAb treatment as previously described.\(^7\) Thymectomized mice that did not receive mAb served as controls to exclude the effect of thymectomy alone in corneal allograft survival. Mice were grafted orthotopically on day 0, and monoclonal antibodies were injected intraperitoneally (0.25 mg/mouse) on days 1, 0, 7, and 14.

Flow Cytometry

The efficacy of in vivo depletion of T-cell subsets with anti-CD4 and anti-CD8 monoclonal antibodies was assessed by flow cytometry. Spleen cells from two similarly treated mice were pooled, washed in Hank's balanced salt solution (HBSS), and incubated for 20 min in 20% horse serum at 0°C. After washing in HBSS, the cells were split into groups for immunoscanning. One group was incubated 30 min at 0°C with either anti-CD4 or anti-CD8 antibodies and washed three times. Both groups then were incubated with secondary, FITC-labeled anti-rat immunoglobulin G for 20 min at 0°C. The cells were washed in HBSS, resuspended in 1% paraformaldehyde, and assayed for positive fluorescence in an Epics Profile Analyzer (Coulter, Hialeah, FL). Gates were set at 1% of total cells, based on staining by secondary antibody alone (<1.0%).

Orthotopic Corneal Allografting

Full-thickness (1.5-mm diameter) penetrating BALB/c corneal grafts were placed onto anesthetized C57BL/6 mice using a procedure described previously.\(^8\) Briefly, the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (1–2 mg/mouse; Abbott, North Chicago, IL). Proparacaine (Alcon, Ft. Worth, TX) was used as a topical anesthetic. A mixture of 1% tropicamide (Alcon) and 2.5% phenylephrine hydrochloride (Alcon) was used to dilate the pupil. A 1.5-mm trephine was used to score the donor cornea before removal of the corneal button using vannas scissors. The recipient cornea was similarly scored with a 1.5-mm trephine and the central 1.5-mm button removed. The donor graft was sewn in place using ten interrupted sutures with 11-0 nylon and a 50-μm diameter needle (Sharpoint; Vanguard, Houston, TX). The anterior chamber was reformed with sodium hyaluronate (Healon; Alcon) or balanced salt solution. Sutures were removed 10 days later. Topical antibiotic (tobramycin; Alcon) was applied immediately after surgery and twice a week thereafter. No immunosuppressive drugs were used either topically or systemically.

Clinical Observations

Grafted mice were observed with a slit-lamp microscope at least twice a week throughout the study period. Graft opacity, edema, and neovascularization were scored as minimal, moderate, or severe as previously described.\(^9\) If all three parameters became moderate or severe more than 7 days after grafting, the graft was recorded as rejected on that day. Hosts were excluded from the study if they had complications such as cataract, anterior chamber synechiae, pupillary block, infection, or loss of the anterior chamber.

Cell-Mediated Cytotoxicity Assay

The mice were killed 15 and 30 days after grafting, and their spleens and lymph nodes were removed using sterile technique. Single-cell suspensions of combined lymph node and spleen cells were prepared as previously described.\(^10\) BALB/c splenic stimulator cells were irradiated (3000 cGy) in a cesium 137 source before in vitro cultivation with C57BL/6 responder lymphoid cells. Responder and stimulator cells (5 × 10^6 of each population in a total volume of 2.0 ml) were added to 24-well trays (Costar, Cambridge, MA) and incubated at 37°C in a humidified CO₂ atmosphere. Effector cells were harvested from boost cultures after 24–48 hr and used as effector cells in chromium-release assays.
Cytotoxicity was determined in a 4-hr chromium 51-release assay as previously reported. Briefly, effector cells were washed and resuspended in complete medium (2 x 10^6 cells/ml) and 100-μl triplicate samples were plated into round-bottomed microtiter plates. Then 100 μl of labeled target cells (10^4 cells/ml) was added to each well. Effector-to-target ratios ranged from 100:1 to 12.5:1. Plates were centrifuged at 100 x g for 2 min and then incubated at 37°C for 4 hr in a humidified 5% CO2 atmosphere. After incubation, the plates were centrifuged for 5 min at 200 x g, and 100 μl of supernatant from each well was removed. The counts per min were determined by a gamma counter (Tracor, Atlanta, GA).

Cytotoxicity was calculated using the formula:

\[
\% \text{ specific release} = \left( \frac{\text{exp. cpm} - \text{spont. release cpm}}{\text{max. release cpm} - \text{spont. release cpm}} \right) \times 100
\]

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

**Skin Grafting**

C57BL/6 mice were immunized with full-thickness BALB/c skin grafts applied to the lateral thorax as previously described. Mice were challenged with orthotopic corneal grafts 3 weeks later.

**Results**

**Orthotopic Corneal Allografting in the Mouse**

The availability of well-defined congenic mouse strains, sophisticated monoclonal reagents, and the large amount of information regarding the murine immune system make the mouse a highly desirable model for studies evaluating the immunobiology of corneal allograft rejection. Accordingly, we used an orthotopic grafting procedure recently described in an effort to establish the fundamental characteristics of orthotopic corneal allografts in mice. For example, to establish the credibility of orthotopic murine corneal grafts, it is important to establish the first and second-set rejection times and the incidence of graft failure in syngrafts. It was also important to be able to distinguish between technical failures and graft rejection. As we might expect, doing successful penetrating keratoplasties on mice was technically difficult. Approximately 30-40% of the orthotopic corneal grafts in all groups failed as a result of surgical and technical complications. Almost all of these failures occurred within the first 7 days of surgery and were the result of cataract formation, synechiae, or infection. Any animals with these complications were excluded from the study. However, despite these technical failures, the feasibility of producing clear penetrating keratoplasties was demonstrated in C57BL/6 mice grafted with syngeneic, C57BL/6 corneal grafts. All syngrafts (n = 5) survived beyond 60 days. By contrast, 83% of the BALB/c corneal allografts were rejected by naive C57BL/6 recipients (median survival time, [MST] 21.2 days. This is equal to the first-set rejection time.), and all preimmunized mice rejected their orthotopic corneal grafts (MST, 16 days or second-set rejection time). The characteristics of murine orthotopic corneal grafts paralleled those reported in a rat keratoplasty model. Grafts that were being rejected became opaque, edematous, and vascularized (Fig. 1). Syngrafts remained clear indefinitely (Fig. 2). Histologic examination of grafts undergoing rejection revealed a heavy infiltration of inflammatory cells, disruption of the stromal architecture, and loss of the endothelial layer (Fig. 3).
Effect of Anti-CD4 and Anti-CD8 Antibodies on Corneal Graft Survival

To determine if anti-CD4 and anti-CD8 mAbs were capable of promoting corneal graft survival, a panel of mice was treated with a mixture of both antibodies. These results showed that the combination of anti-CD4 and anti-CD8 greatly reduced the rejection rate and significantly prolonged the MST of those grafts that did undergo rejection (Table 1). Treatment with anti-CD8 antibody alone did not reduce the incidence of rejection compared with naive controls, although treated mice did have a modest, but significant prolongation of MST (Table 1). By contrast, anti-CD4 antibody prevented graft rejection in four of six animals and thus mimicked the effect of combined treatment with both antibodies.

Flow-cytometric analysis of spleen cells isolated from antibody-treated animals indicated that CD4-positive and CD8-positive T-cells were depleted selec-
Table 1. Effect of in vivo antibody treatment on corneal allograft survival

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. rejected/no. grafted*</th>
<th>MST†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngeneic</td>
<td>0/5 (0%)</td>
<td>(&gt;60 days)</td>
</tr>
<tr>
<td>Allogeneic control</td>
<td>5/6 (83%)</td>
<td>21.2 ± 1.5</td>
</tr>
<tr>
<td>Preimmune allogeneic</td>
<td>6/6 (100%)</td>
<td>16.2 ± 2.5</td>
</tr>
<tr>
<td>Anti-CD4 treated</td>
<td>2/6 (33%)</td>
<td>25.0 ± 0.0</td>
</tr>
<tr>
<td>Anti-CD8 treated</td>
<td>5/6 (83%)</td>
<td>26.4 ± 4.0</td>
</tr>
<tr>
<td>Anti-CD4/CD8 treated</td>
<td>2/7 (29%)</td>
<td>26.5 ± 1.0</td>
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* Any animals developing any surgery-associated complications during the first 7 days following transplantation were excluded.
† MST = median survival time.

greatly reduced the incidence of corneal graft rejection and prolonged the MST. To our surprise, anti-CD8 mAb did not promote corneal graft survival even though this antibody eliminated the host’s allospecific cytotoxic T-lymphocyte (CTL) responses. This is particularly puzzling in light of previous studies that found that the rejection of orthotopic corneal allografts in rats was associated with the development of specific CTLs and occurred in the absence of detectable delayed-type hypersensitivity (DTH). Moreover, our laboratory repeatedly showed that heterotopic and orthotopic corneal allografts consistently do not elicit allospecific DTH responses either before, during, or after rejection. Although the anti-CD8 mAb treatment appeared to eliminate in vitro CTL activity, it is possible that residual activity remained in vivo. The remaining CD4-positive T-cells in the anti-CD8-treated animals may mediate direct cytotoxic effects not detectable in the 4-hr chromium-release assay. Alternatively, graft rejection might be mediated by second-level effector cells, such as macrophages, activated by cytokines released from antigen-specific CD4-positive T-cells.

Table 2. Depletion of T cell subsets by in vivo treatment with anti-CD4 and anti-CD8 monoclonal antibodies

<table>
<thead>
<tr>
<th>Antibody treatment</th>
<th>% Positively staining spleen cells*</th>
</tr>
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<tbody>
<tr>
<td>CD8+</td>
<td>CD4+</td>
</tr>
<tr>
<td>GK 1.5</td>
<td>21.8</td>
</tr>
<tr>
<td>YTS 169.4</td>
<td>0.5</td>
</tr>
<tr>
<td>GK 1.5/YTS 169.4</td>
<td>0.5</td>
</tr>
<tr>
<td>None</td>
<td>16.0</td>
</tr>
</tbody>
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* Expression of CD4 and CD8 surface determinants assessed by flow cytometry, as described in Materials and Methods. Assay was performed on antibody treated animals 14 days after in vivo administration of antibody.
Many studies examined the role of CD4-positive and CD8-positive T-cells in the rejection of skin allografts. Collectively, the data from several studies suggest that, depending on the histocompatibility disparity under investigation, either CD4-positive or CD8-positive cells can play a major role in skin allograft rejection. However, in the case of disparity in the entire MHC plus many minor H loci, neither CD4 nor CD8 antibody treatment alone significantly prolonged skin graft survival. Others used adoptive transfer of purified T-cell subsets to nude recipients and showed that either CD8-positive or CD4-positive T-cells were capable of rejecting whole MHC-mismatched grafts. The whole issue of whether CTL or DTH functions are restricted to CD8-positive or CD4-positive T-cell populations is more semantic than real because CD4-positive T-cells can mediate class II restricted CTL activity and CD8-positive T-cells can produce DTH lesions. In our study, it was surprising that corneal allograft rejection was prevented by treatment with anti-CD4 antibody alone. However, it is possible that class II MHC-restricted CD4-positive CTLs played an important role in mediating graft destruction; we recently demonstrated that the orthotopic grafting procedure itself induces transient, but strong, expression of donor class II MHC antigens on corneal allografts. The failure of the CD8-positive cells to produce cytolytic effects in the anti-CD4 treated mice probably was a result of the absence of CD4-positive helper T-cells.

In conclusion, our results indicate that systemic treatment with anti-CD4 antibody is an effective method for promoting corneal allograft survival against highly immunogenic corneal allografts. These findings also indicate that CD8-positive T-cells are not necessary or sufficient to mediate corneal allograft rejection alone.

**Key words:** corneal graft, keratoplasty, mouse, antibody

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**References**