Rat Corneal Allograft Survival Prolonged by the Superantigen Staphylococcal Enterotoxin B

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PURPOSE. The purpose of this study was to determine the optimal conditions for prolonging corneal allograft survival by inducing anergy with the superantigen staphylococcal enterotoxin B (SEB).

METHODS. A rat model of penetrating keratoplasty, whereby Fisher344 donor corneas are implanted into Lewis recipients, was used to evaluate the effects of SEB on inducing immune-mediated allograft rejection. To induce anergy, SEB was injected into the peribulbar space of Lewis rats. Furthermore, histopathology and immunofluorescent staining were used to examine the levels of infiltrating CD4+ and CD8+ T lymphocytes and NK1.1+ lymphocytes.

RESULTS. By administering SEB, at doses of 90 or 120 μg/kg 7 days before and after keratoplasty, we suppressed the episode of corneal graft rejection for a median of 12 and 30 days, respectively. In contrast, rejection was observed when 30 or 60 μg/kg of SEB was administered. After SEB injections, lymphocyte infiltration into the corneal grafts was reduced, and the expression of NK1.1+ lymphocytes was enhanced, suggesting that anergy may be occurring. Also, there were no differences in the number of infiltrating CD4+ and CD8+ T lymphocytes cells between the control group and groups injected with 30 and 120 μg/kg SEB on postoperative days 10 and 30.

CONCLUSIONS. Inducing anergy with the superantigen SEB prolonged corneal graft survival in a rat model of penetrating keratoplasty. Therefore, these results support the possibility of prolonging corneal allograft survival in a clinical setting by preventing immune-mediated rejection through the administration of the superantigen SEB. (Invest Ophthalmol Vis Sci. 2003;44:3546–3551) DOI:10.1167/iovs.02-0845

Corneal transplantation is the most common and successful form of solid tissue transplantation. Although the rejection rate for routine keratoplasty is only 10% in the first year, there are a significant number of corneal graft failures in high-risk cases of keratoplasty due to immune-mediated rejection.1,2 Corticosteroids and cyclosporin A greatly reduce the rejection rate of corneal allografts, but high doses and/or long-term administration of these drugs can produce deleterious side effects, such as glaucoma, cataract formation, nephrotoxicity, hypertension, and hepatotoxicity.3

The term superantigen (SAg) is used to describe those microbial products that activate a large portion of the T-cell population (5%–50%), whereas conventional antigens stimulate only 0.01%. Superantigens differ from conventional antigens in that they bind to the outside of the peptide-binding groove of MHC class, thus exerting their effects as an intact molecule without being processed. Furthermore, recognition of SAgS by the T-cell receptor (TCR) depends only on the variable region of the TCR β chain (Vβ). Therefore, SAgS stimulate both antigen-presenting cells (APCs) and T lymphocytes, which leads to the massive production of cytokines, enhanced expression and/or activation of cell adhesion molecules, T-cell proliferation, activation-induced apoptosis, and T-cell anergy.4 A study has shown that injecting the SAg staphylococcal enterotoxin B (SEB) into mice produces transient, rapid hyperactivation and proliferation of T cells, which are eliminated by activation-induced cell death within 48 hours.5 In addition, the remaining SAg-reactive cells fail to proliferate in response to a secondary SAg challenge. The proliferative unresponsiveness of the secondary SAg responder T cells has been termed anergy. However, on subsequent analysis, secondary SAg responder T cells may not be truly anergic because they could be reacting to the second SAg exposure.6 Wang et al.7 showed that CD8+ regulatory suppressive T cells could enforce anergy by inhibiting cell division of preactivated T cells, not by the SAg response of naïve T cells.

Damage to healthy, transplanted tissue can be curtailed by reducing the inflammatory response of the immune system. Our studies have shown that the bacterial superantigen SEB can inhibit the rejection of transplanted mouse bone marrow cells and peripheral lymphocytes, suggesting that injections of SEB may induce peripheral anergy to allogeneic organ grafts. In addition, CD4+ T cells appear responsible for maintaining this anergy rather than the CD8+ T cells.8 Our studies confirmed that the induction of transplantation anergy by SEB injections contribute to hematopoietic chimerism, defined as the coexistence of host and donor cells, and that mixed lymphocyte reactions (MLRs) are significantly low. This in vivo SEB-induced anergy may be associated with the clone deletion of T-helper (Th)1 cells. The specific dosage of SEB is critical because, if the dosage is too high or too low, then anergy may not develop in the injected mice.8,9

Several mechanisms that can induce CD4+ T-cell death or unresponsiveness have been identified. These mechanisms include T-cell anergy, which is due to the absence of costimulation at the time of activation, and Fas-mediated activation-induced cell death (AICD). Cytokines, such as IFN-γ, TNF-α, and IL-10, can also mediate T-cell suppression; however, the detailed mechanisms involved in the induction of these cytokine-regulated T-cell death pathways have not been fully characterized.10

Bacterial SAgS are a large group of polypeptides that are produced by bacterial strains, such as Staphylococcus aureus and S. pyogenes. SAgS have been implicated in the pathogenesis of toxic-shock-like syndromes in both animal models and in humans. In particular, the in vivo immune response to the S. aureus SAg SEB represents a useful model for studying in vivo cytokine regulation and the phenomenon of peripheral T-cell
unresponsiveness. After their initial clonal expansion and cytokine production, SEB-reactive T cells are thought to become unresponsive to further TCR stimulation.12–13 T-cell deletion and unresponsiveness could be induced by oral administration or intrathymic injection of low doses of SEB, which could result in not only gut-associated lymphoid tissue (GALT) and thymic alterations but also peripheral pivotal immune alterations.12,13

The SEB used in this study was produced from S6 cocci ferment, which has a molecular mass of 2.84 to 2.9 kDa and an isoelectric point of 8.6, and purified by affinity chromatography. In our previous experiments, SEB selectively decreased the percent of CD4+ T cells and CD4+ T/H2Kb+, cells, but had no effect on the number of CD8+ cells after allogenic mouse cell transplantation. Accordingly, the proliferative response caused by MLRs in the recipient mouse decreased significantly.8 Herein, we report that treating an inbred rat model of allograft keratoplasty rejection with SEB injections into the peribulbar space at preoperative and postoperative day 7 led to rat immune unresponsiveness (anergy), which persisted for 15 to 20 days after only one injection. Because SEB entered the bloodstream and induced CD4+ and CD8+ T lymphocyte unresponsiveness, it appears that the effect was systemic.8 In all, we used the superantigen SEB to induce anergy for the purpose of prolonging corneal allograft survival and to observe any subsequent immune reactions.

**Materials and Methods**

**Animals**

Adult inbred female F344 and Lew rats were purchased from the Animal Institute of Chinese Medical Academy (Beijing, China) and used as experimental subjects between 2 and 2.5 months of age. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction of Anergy**

The SAg, staphylococcal enterotoxin B (SEB; Chinese Patent No. 01103991.4) was used for inducing anergy. SEB, at doses of 30, 60, 90, or 120 µg/kg were injected into the peribulbar space of each Lew inbred rat 7 days before and after keratoplasty. Thirty-six Lew rats were divided into six groups as follows: group 1 F344/Lew (control, 0.2 mL saline buffer, peribulbar); group 2 F344/Lew (SEB, 30 µg/kg body weight, peribulbar); group 3 F344/Lew (SEB, 60 µg/kg body weight, peribulbar); group 4 F344/Lew (SEB, 90 µg/kg body weight, peribulbar); group 5 F344/Lew (SEB, 120 µg/kg body weight, peribulbar); and group 6 Lew/Lew (no treatment).

**Orthotropic Corneal Transplantation**

F344 corneal grafts (3 mm diameter) were transplanted orthotopically into anesthetized Lew eyes by a procedure described by Ma et al.2 Both the donor graft and the recipient graft bed were scored with 3- and 2.5-mm trephines. The donor graft was sewn into place with eight interrupted 10-0 nylon (Alcon Laboratories, Fort Worth, TX) sutures. To protect the transplant, a blepharorrhaphy was attached by means of interrupted 10-0 nylon (Alcon Laboratories, Fort Worth, TX) sutures.

**Assessment of Graft Survival**

After surgery, all rats were subjected to clinical examinations by slit lamp microscopy every day for 2 weeks followed by twice a week thereafter. The transplants were evaluated using a modified form of a previously described scoring system. The scoring system took into account opacity, edema, and newvascularization14 (Table 1). An immune-mediated rejection episode was considered to occur if the combined score of all three factors was equal to or exceeded 6. Also, the mean survival time for each group was calculated.

**Histopathological and Immunofluorescence Evaluation**

At postoperative days 10 and 30, two graft-recipient animals were killed by carbon dioxide inhalation. Afterward, the eyes were enucleated and fixed in a buffered formalin solution (4%). For histologic assessment, the formalin-fixed eyes were cut into 4-µm-thick sections and subjected to hematoxylin and eosin staining. For immunofluorescence evaluation, the 4-µm-thick sections were subjected to direct immunofluorescence using the monoclonal antibodies OX-35 (Cy-Chrome anti-rat CD4, cat. 554839), OX-8 (FITC anti-rat CD8a, cat. 554865), and RPE-conjugated mouse anti-rat CD161a (NKRP1A; all from BD Biosciences, Lincoln Park, NJ), which are markers for CD4+ and CD8+ T cells and NK1.1+ lymphocytes, respectively.

**Statistical Analysis**

The mean survival time (MST) and the clinical scoring data were compared between the various groups by means of one-way ANOVA and the independent-sample t-test, on computer (SPSS for Windows, ver. 10.0; SPSS Science, Chicago, IL).

**Results**

**Clinical Evaluation**

Transplanted grafts, which exhibited opacity due to operative errors, such as bleeding, suture dehiscence, and/or lens opacification, within the first 3 days after surgery were omitted from the statistics (one rat in groups 2, 4, and 5 and two rats in group 3). On postoperative day 10, all grafts from groups 1 to 3 exhibited rejection episodes (Figs. 1A–C). The grafts exhibited severe edema and infiltration into the epithelium and stroma, and newly formed vessels began to penetrate the transplanted grafts. However, in groups 4 and 5, almost all grafts showed no indications of rejection and no alteration in their normal, physiological transparency (Figs. 1D, 1E). Accordingly, isografts from group 6 showed no immunologic reaction (Fig. 1F). On postoperative day 30, all grafts from groups 1 to 3 exhibited scarring (Figs. 2A–C, respectively), several grafts from groups 4

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**Table 1. Clinical Scoring Scheme for the Severity of Corneal Graft Rejection**

<table>
<thead>
<tr>
<th>Type/Score</th>
<th>Clinical Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No opacity</td>
</tr>
<tr>
<td>1</td>
<td>Slight opacity, details of iris clearly visible</td>
</tr>
<tr>
<td>2</td>
<td>Some details of iris no longer visible</td>
</tr>
<tr>
<td>3</td>
<td>Pronounced opacity, pupil still recognizable</td>
</tr>
<tr>
<td>4</td>
<td>Total opacity</td>
</tr>
<tr>
<td>0</td>
<td>No edema</td>
</tr>
<tr>
<td>1</td>
<td>Mild edema</td>
</tr>
<tr>
<td>2</td>
<td>Pronounced edema with raised transplant</td>
</tr>
<tr>
<td>3</td>
<td>Pronounced edema with small bleb</td>
</tr>
<tr>
<td>4</td>
<td>Pronounced edema with large bleb</td>
</tr>
<tr>
<td>0</td>
<td>No vessels</td>
</tr>
<tr>
<td>1</td>
<td>Vessels appearing in the corneal bed</td>
</tr>
<tr>
<td>2</td>
<td>Vessels appearing in the graft periphery</td>
</tr>
<tr>
<td>3</td>
<td>Vessels extending deeper</td>
</tr>
<tr>
<td>4</td>
<td>Vessels extending to the center</td>
</tr>
</tbody>
</table>

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**Enhancement of Corneal Allograft Survival by SEB** 3347
and 5 exhibited no immune-mediated rejection (Figs. 2D, 2E), and all grafts from group 6 remained transparent (Fig. 2F).

Isografts survived for a median of more than 30 days (Table 2); however, allografts underwent clinical rejection at a median of 4 to 7 days after keratoplasty. The average transplant survival rate of the allogenic group (group 1, F344/Lewis) was 5.17 ± 1.83 days (SD). Lewis rats receiving injections of 30 μg/kg SEB (group 2) and 60 μg/kg SEB (group 3) exhibited an MST of 7 days, which was not a statistically significant (P > 0.05) elongation of transplant survival when compared with the control (group 1).

However, the administration of 90 μg/kg SEB (group 4) and 120 μg/kg SEB (group 5) exhibited an MST of 12 and 30 days, respectively, which was a statistically significant (P < 0.01) elongation of transplant survival when compared with the control (group 1). Figure 3 shows the mean clinical scores of all groups after graft transplantation. All the rats in groups 1 to 3 were defined as rejecting the transplant after 10 days. The mean rejection index (RI) was decreased to statistically significant levels in the grafts from groups 4 and 5 when compared with the control (P < 0.01). The mean opacity, edema, and neovascularization scores diminished in the allografts from groups 4 and 5 when compared with the control, which was statistically significant (P < 0.01). The mean opacity scores for groups 4 to 6 reached similar levels, but they were lower than those of groups 1 to 3 after postoperative day 30. Also, the corneal edema and neovascularization scores exhibited a similar pattern (Figs. 3A–D).

**Histopathological and Immunofluorescent Staining**

At postoperative day 10, the grafts from the control group (group 1) exhibited pronounced edema, characterized by an...
extreme thickening of the stroma, particularly in the area of the epithelial basal membrane. Also, pronounced mononuclear infiltration was present in all layers of the corneal grafts and more pronounced in the stroma and deep layers of the epithelium. A dense mononuclear infiltrate and an accumulation of macrophages were observed at the edge of the transplant and near the interrupted suture (Fig. 4A).

Similar results were observed in grafts from groups 2 and 3; however, the inflammatory infiltration was reduced slightly (Fig. 4B). In contrast, no significant infiltration of lymphocytes was observed in grafts from groups 4 and 5, especially from group 5 (Fig. 4C). However, at postoperative day 30, there were no significant differences between all the transplant groups (Figs. 4D–F).

Immunofluorescent staining for rat CD4\(^{+}\) and CD8\(^{+}\) T cells, using the monoclonal antibodies OX35 and OX8, respectively, revealed that there were no differences in the amount of infiltrating CD4\(^{+}\) and CD8\(^{+}\) T lymphocytes between all groups at postoperative days 10 and 30 (data not shown).

However, immunofluorescent staining for NK1.1\(^{+}\) lymphocytes, using the monoclonal antibody R-PE-conjugated mouse anti-rat CD161a (NKR-P1A) showed significant differences in the rat corneal epithelium and central stroma of the control group and the 120 \(\mu\)g/kg SEB–injected group on postoperative days 10 and 30 (Fig. 5).

**DISCUSSION**

Although corneal grafts have an excellent success rate compared with other types of transplants, 10%–20% fail as a result of immunologic rejection. In humans, high-risk keratoplasty
FIGURE 4. Histopathology of corneal grafts by hematoxylin-eosin staining. On postoperative day 10: (A) group 1, allograft control; (B) group 2, 30 μg/kg SEB; and (C) group 5, 120 μg/kg SEB. On postoperative day 30: (D) group 1, allograft control; (E) group 2, 30 μg/kg SEB; and (F) group 5, 120 μg/kg SEB. Magnification, ×100.

FIGURE 5. Expression of NK1.1⁺ lymphocytes in rat transplants by immunofluorescent staining. On postoperative day 10: (A) group 1, allograft control and (B) group 5, 120 μg/kg SEB. On postoperative day 30: (C) group 1, allograft control and (D) group 5, 120 μg/kg SEB. Magnification, ×100.
resulting in immune-mediated rejection remains the leading cause of allograft failure.\textsuperscript{1,2} Besides blood type (ABO) or major histocompatibility complex (MHC) matching, immunosuppressive agents such as steroids, cyclosporin A, FK506, IL-1ra, CTLA-4Tg, and anti-CD4 monoclonal antibodies have been used for treating allograft rejection.\textsuperscript{15–20} However, more-effective, less-toxic immunosuppressive agents are still needed to prevent immune-mediated corneal transplant rejection.

The mechanism of tolerance induced by SEB is poorly understood. Immunity studies have shown that peripheral deletion in response to soluble Ag is one mechanism by which the immune system eliminates self-reactive T cells that escape thymic deletion. Experimental models, in which SAgS are injected into normal mice or relevant peptide Ags are injected into TCR transgenic mice, have provided evidence for peripheral deletion.\textsuperscript{52} Another potential mechanism for silencing T cells is functional inactivation, often referred to as anergy. Based largely on in vitro experiments with Th1 clones, anergy is defined as a defect in TCR-dependent proliferation that is acquired as a result of prior TCR stimulation in the absence of APC-derived costimulatory signals or proliferation.\textsuperscript{22}

Our study evaluated the potential use of SEB as a strategy for preventing corneal graft rejection. The SEB used in our experiment prolonged rat graft survival by reducing lymphocyte infiltration, edema, and neovascularization. Because graft rejection is typically due to corneal inflammation, we used corneal opacity as a marker of immune-mediated rejection. The data in Figure 3B show that the scores of corneal opacity in groups 4 and 5 were equal to or below group two and less than groups 1 and 3. Because the number of rats in each group were different (especially group 5) and because dexamethasone was used in our experiments during the first three postoperative days, more experiments should be performed to verify that SEB plays a role in avoiding corneal allograft rejection through the induction of anergy.

Also, the infiltration of inflammatory lymphocytes into grafts in group 5 (treated by SEB 120 μg/kg, peribulbar) was reduced. The expression of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells was unchanged after SEB injection in all rat corneal grafts; however, there were more NK1.1\textsuperscript{+} lymphocytes present in the corneal grafts from group 5 (120 μg/kg SEB) than in control grafts. These results suggest that T cells were unresponsive;\textsuperscript{53} but the absence of an immune response must be confirmed. In addition, more work is needed to help explain further the unchanged expression of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and the increased expression of NK1.1 T cells in the rat corneal grafts.

In summary, these findings suggest the feasibility of using SEB-induced anergy as a means of reducing corneal allograft rejection. Whether administration of SEB can enhance allograft tolerance and permit the use of a less-intensive postoperative treatment for patients should be verified further. The remarkable capacity of SEB to prevent rejection in an otherwise high-risk setting suggests that SEB may be useful as a treatment in high-risk keratoplasty in humans; however, well-controlled clinical trials are needed to confirm this assertion.

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References


