and homozygous ACD patients is caused by the difference in age and accumulated layers of deposition.

Previously, corneal dystrophies have been classified by clinical and histopathologic findings. Therefore, differential diagnosis of corneal dystrophy is complicated. Our results suggest that in the future, corneal dystrophies should be classified according to their genotype.

References

Cytokine Expression during Orthotopic Corneal Allograft Rejection in Mice
Yoichiro Sano, Hideya Osawa, Chie Sotozono, and Shigeru Kinoshita

Purpose. The acquisition of cell-mediated immunity against donor antigens has been shown to be associated with rejection of orthotopic corneal allografts, but the mechanisms that cause corneal allograft destruction in grafted tissue remain obscure. To determine which T-cell subsets infiltrate graft tissue and cause graft rejection, cytokine expression was examined in corneal tissue after orthotopic corneal allograft.

Methods. BALB/c mice received orthotopic corneal allografts from either syngeneic BALB/c or allogeneic C57BL/6 donors. At 1 or 4 weeks after grafting, the mice were euthanized, and their corneas were removed. Corneal tissue was frozen, homogenized, and placed in phosphate-buffered saline (PBS). Each sample consisted of five corneas in 500 ml PBS. After centrifugation, the supernatant was collected, and the concentration of the following cytokines was measured by enzyme-linked immunosorbent assay: interleukin (IL)-1α, IL-2, IL-4, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α.

Results. Significantly increased amounts of proinflammatory cytokines (IL-1α and TNF-α) were detected in supernatants from all grafted corneas (both syngeneic and allogeneic) at 1 week after grafting. At 4 weeks after grafting, supernatants from normal corneas, corneas with syngeneic grafts, and corneas with accepted corneal allografts contained undetectable amounts of IL-2 and IFN-γ, whereas supernatants from corneas with rejected corneal allografts contained significant amounts of IL-2 and IFN-γ. There were no significant differences in the amounts of IL-4 or IL-10 among all samples. Histologic examination confirmed the expression of IL-2 and IFN-γ in rejected corneal allografts.

Conclusions. Because IL-2 and IFN-γ are secreted primarily by T-helper type 1 (TH 1) cells, whereas IL-4 and IL-10 are secreted by T-helper type 2 (TH 2) cells, these results indicate that TH 1-type cytokines, rather than TH 2-type cytokines, contribute to the rejection of orthotopic corneal allografts in graft tissue. (Invest Ophthalmol Vis Sci. 1998;39:1953-1957)

Study of the immune response in orthotopic corneal transplantation has revealed unique features associated with the anterior segment of the eye, compared with other types of organ transplantation. Unlike other types of organ transplants, corneal grafts that display major histocompatibility complex class I or class II alloantigens only are less likely to be rejected, whereas grafts that display only minor transplantation antigens are rejected much more frequently.1,2 It has been shown also that the acceptance of orthotopic murine corneal allografts correlates positively with the development of donor-specific anterior chamber-associated immune deviation.3 Moreover, recent studies have reported that donor-specific delayed hypersensitivity responses detected after orthotopic corneal allografts are directed at donor-minor alloantigens, but not at major histocompatibility complex alloantigens.4

Using experimental animals, Callanan et al5 performed a study to characterize the mechanisms of orthotopic corneal allograft rejection and reported a correlation between rejection and cell-mediated immunity in the recipients. Although this study revealed the acquisition of systemic cell-mediated immu-
The donor cornea was then placed in the recipient bed and after corneal transplantation, grafts were examined by slit lamp. Transplants were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

MATERIALS AND METHODS

Mice

Six- to 8-week-old BALB/c and C57BL/6 mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Transplantation and Grafting

Orthotopic corneal transplantation was performed as described previously. Briefly, donor central corneas (2 mm in diameter) were excised using Vannas scissors and placed in chilled phosphate-buffered saline (PBS). Animals were anesthetized with intraperitoneal injections of ketamine (3–4 mg/animal) and xylazine (0.1 mg/animal). The graft bed was prepared by exciting with Vannas scissors a 2-mm site in the central cornea of the right eye. The donor cornea was then placed in the recipient bed and secured with eight interrupted sutures (11-0 nylon). All grafted eyes were examined after 72 hours; at that time, grafts with technical difficulties (hyphema, infection, or loss of the anterior chamber) were excluded from further consideration. At 9 days after grafting, all sutures were removed.

Evaluation and Scoring of Orthotopic Corneal Transplants

After corneal transplantation, grafts were examined by slit lamp microscopy at weekly intervals. Each time, grafts were scored for opacity and neovascularization. A scoring system was devised to describe in semiquantitative terms the extent of opacity (0–5+), as described previously. Grafts with opacity scores of 2+ or greater at 8 weeks were considered to have been rejected.

Cytokine Quantitation

The cytokines in corneal tissues were quantified as described previously. Corneas (4 mm in diameter) were excised from the limbus at 1 or 4 weeks after grafting. Each test sample was comprised of five corneas. The samples were frozen with liquid nitrogen and then homogenized in chilled PBS at a ratio of 100 μL/ml per cornea. The supernatants were collected by centrifugation at 1500g for 10 minutes and stored at −80°C until used. Cytokine production levels were measured using enzyme-linked immunosorbent assay systems for IL-1α, tumor necrosis factor (TNF-α) (Genzyme, Cambridge, MA), IL-2, IL-4, IL-10, and IFN-γ (Endogen, Boston, MA). The limits of detection were 15 pg/ml for IL-1α and TNF-α, 3 pg/ml for IL-2, 5 pg/ml for IL-4, 0.14 U/ml for IL-10, and 15 pg/ml for IFN-γ. In all enzyme-linked immunosorbent assay experiments, recombinant murine cytokines were used as positive controls. At least three samples were tested in each group, and statistical analysis was performed on their values using the Student's t-test.

Immunohistochemical Analysis

Based on the results of the enzyme-linked immunosorbent assays, immunohistochemical analyses were performed to determine the expression of cytokines in the tissue. Corneas were fixed with formaldehyde in 0.1 M PBS and then dehydrated with 30% sucrose obtained 4 weeks after grafting were fixed with 4% paraformaldehyde in 0.1 M PBS and then dehydrated with 20% sucrose. For 16 hours at 4°C, incubated further with biotinylated anti-goat or anti-rat IgG (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature, and then incubated in avidin-biotin-peroxidase complex (ABC; Vector Laboratories, 1:100) for 90 minutes at room temperature. To detect immune complexes, the sections were immersed for 10 minutes at room temperature in 0.02% 3, 3′-diaminobenzidine in 100 ml of 0.05 M Tris-HCl buffer (pH 7.6) containing 20 μL of 30% H2O2. Between each step, the sections were thoroughly washed in 0.1 M PBS. Corneal sections were also stained with hematoxylin and eosin. In all assays, negative controls were prepared using 20 μg/ml goat or rat IgG for the primary antibody.

RESULTS

Appearance of Orthotopic Corneal Allografts and Isografts

Ten BALB/c mice received orthotopic corneal isografts from BALB/c donors, and 10 BALB/c mice received orthotopic corneal allografts from C57BL/6 mice. As previously reported, all grafts, isografts and allografts, showed slight edema and opacity at 1 week after grafting; however, all grafts appeared to be clear at 2 weeks after grafting. At 4 weeks after grafting, all isografts appeared completely clear. Five corneal allografts of 10 (50%) developed moderate to severe opacity approximately 4 weeks after grafting and were considered to have been rejected, whereas the other 5 corneal allografts (50%) remained clear and were considered to have been accepted.

Proinflammatory Cytokine Production in Corneal Tissue after Grafting

Because IL-1α and TNF-α are known as proinflammatory cytokines, which cause nonimmune-mediated inflammatory response, the secretion of proinflammatory cytokines was studied at 1 week after grafting, when all grafts (isografts and allografts) showed mild edema and opacity, and at 4 weeks, when some of the allografts had been rejected. The supernatants from normal BALB/c corneas contained 4.0 ± 0.5 pg/cornea of IL-1α and 8.9 ± 0.9 pg/cornea of TNF-α. The supernatants from corneal tissue with isografts contained significantly increased amounts of IL-1α (7.5 ± 0.3 pg/cornea; P = 0.04) and TNF-α (15.2 ± 2.4 pg/cornea; P = 0.04) as did those from corneal tissue with allografts (5.6 ± 0.3 pg/cornea, P = 0.02, and 13.5 ± 1.4 pg/cornea, P = 0.02, respectively) at 1 week after grafting. IL-1α and TNF-α were
TABLE 1. Cytokine Concentrations Found in Corneal Tissue at 1 Week after Grafting

<table>
<thead>
<tr>
<th>Proinflammatory</th>
<th>IL-1α (pg/cornea)</th>
<th>TNF-α (pg/cornea)</th>
<th>IL-2 (pg/cornea)</th>
<th>IFN-γ (pg/cornea)</th>
<th>IL-4 (pg/cornea)</th>
<th>IL-10 (U/cornea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cornea†</td>
<td>4.0 ± 0.5</td>
<td>8.9 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>4.6 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Cornea with isograft‡</td>
<td>7.5 ± 0.3*</td>
<td>15.2 ± 2.4*</td>
<td>ND</td>
<td>ND</td>
<td>5.0 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Cornea with allograft§</td>
<td>7.1 ± 0.7*</td>
<td>13.5 ± 1.4*</td>
<td>ND</td>
<td>ND</td>
<td>6.0 ± 0.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Th 1/Th 2, T-helper type 1 or type 2 cells, respectively; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; ND, not detected.
* Mean value is significantly greater than normal cornea.
† Normal corneas were obtained from naive BALB/c mice.
‡ Corneas obtained from BALB/c mice with syngeneic BALB/c corneal grafts.
§ Corneas obtained from BALB/c mice with allogeneic C57BL/6 corneal grafts.

At 4 weeks after grafting, significantly increased amounts of IL-1α were detected only in supernatants from corneal tissue with rejected allografts (P = 0.02). No increase in the amount of TNF-α was detected in any of the samples (Table 1).

Th 1 and Th 2 Cytokine Production in Corneal Tissue after Grafting

Because Th 1 cells secrete IL-2 and IFN-γ, whereas T-helper type 2 (Th 2) cells secrete IL-4 and IL-10, the amounts of these cytokines were quantified in corneal tissue to examine which cytokines were most abundant during corneal allograft rejection. IL-2, IFN-γ, and IL-10 were not detected in the supernatants from normal corneal tissue. The supernatants from corneal tissue with either isografts or allografts at 1 week after grafting contained undetectable amounts of IL-2, IFN-γ, and IL-10. However, IL-4 was detected in the supernatants from corneal tissue with isografts (5.0 ± 0.3 pg/cornea) and allografts (6.0 ± 0.4 pg/cornea), but the amounts were not significantly different from those in normal BALB/c corneal tissue (4.6 ± 0.6 pg/cornea) (Table 2).

At 4 weeks after grafting, significantly increased amounts of IL-2 (6.4 ± 4.5 pg/cornea; P = 0.01) and IFN-γ (12.0 ± 0.3 pg/cornea; P = 0.001) were detected in the supernatants of corneal tissue with rejected allografts, whereas supernatants from corneal tissue with either isografts or accepted allografts contained undetectable amounts of IL-2 and IFN-γ. IL-10 was not detected in any of the supernatants. IL-4 was detected in supernatants from corneal tissue with isografts (5.9 ± 0.4 pg/cornea), accepted allografts (4.7 ± 1.0 pg/cornea), and rejected allografts (4.8 ± 0.8 pg/cornea), but levels were not significantly different from those in normal BALB/c corneas (Table 2).

Immunohistochemical Analysis

Because significant amounts of IL-2, IFN-γ, and IL-1α were detected, subsequent experiments were designed to examine the origin of these cytokines in rejected corneal allografts. The rejected graft tissues were stained with anti-IL-2, anti-IFN-γ, and anti-IL-1α antibodies using avidin-biotin-peroxidase complex assays. In the rejected corneal tissues, strongly positive staining for IL-2 and IFN-γ was observed in the stroma (Fig. 1A, 1B). Positive staining for IL-1α was also found in the epithelium and stroma of rejected tissue, whereas weakly positive staining was found only in the epithelium of accepted tissue (Figs. 1C, 1D).

DISCUSSION

Previous reports in which laboratory animals were used have indicated that cell-mediated immunity plays a significant role in corneal allograft rejection. Using rat corneal transplantation models, Callanan et al.² have shown that rejection of ortho-

TABLE 2. Cytokine Concentrations Found in Corneal Tissue at 4 Weeks after Grafting

<table>
<thead>
<tr>
<th>Proinflammatory</th>
<th>IL-1α (pg/cornea)</th>
<th>TNF-α (pg/cornea)</th>
<th>IL-2 (pg/cornea)</th>
<th>IFN-γ (pg/cornea)</th>
<th>IL-4 (pg/cornea)</th>
<th>IL-10 (U/cornea)</th>
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<tr>
<td>Normal cornea†</td>
<td>4.0 ± 0.5</td>
<td>8.9 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>4.6 ± 0.6</td>
<td>ND</td>
</tr>
<tr>
<td>Cornea with isograft‡</td>
<td>6.0 ± 0.9</td>
<td>8.1 ± 0.8</td>
<td>ND</td>
<td>ND</td>
<td>5.9 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>Cornea with accepted allograft§</td>
<td>5.2 ± 0.4</td>
<td>10.3 ± 0.9</td>
<td>ND</td>
<td>ND</td>
<td>4.7 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>Cornea with rejected allograft</td>
<td></td>
<td></td>
<td>10.5 ± 0.7*</td>
<td>9.8 ± 1.1</td>
<td>16.4 ± 4.5*</td>
<td>12.0 ± 0.3*</td>
</tr>
</tbody>
</table>

Th 1/Th 2, T-helper type 1 or type 2 cells, respectively; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; ND, not detected.
* Mean value is significantly greater than normal cornea.
† Normal corneas were obtained from naive BALB/c mice.
‡ Corneas obtained from BALB/c mice with syngeneic BALB/c corneal grafts.
§ Corneas obtained from BALB/c mice with accepted allogeneic C57BL/6 corneal grafts.
FIGURE 1. Immunohistochemical staining of corneal allografts at 4 weeks after grafting. Interleukin (IL)-2-positive cells (A) and interferon (IFN)-γ-positive cells (B) are present in the rejected corneal allograft. Strong staining for IL-1α can be seen in the epithelium and stroma of rejected corneal tissue (C), whereas IL-1α staining is slight in the epithelium of accepted corneal tissue (D). Bar, 100 μm.

topic corneal allografts is mediated primarily by cytotoxic T cells. Data from Joo et al.7 indicated that donor-specific DH, rather than CTL, is associated with corneal allograft rejection in mice. Although these reports suggest a correlation between corneal allograft rejection and systemic donor-specific cell-mediated immunity, the mechanisms of graft rejection remain uncertain in corneal tissue. It has been suggested that the anterior segment of the eye, including the cornea itself, is an immunologically privileged site. Cousins et al.8 have shown that intraocular DH was suppressed when Mycobacterium tuberculosis antigens were injected into the anterior chamber of the eyes of mice that had been systemically immunized with M. tuberculosis antigens previously. Ksander and Streilein9 have shown that cytotoxic T cells directed at the alloantigens of intraocular tumors could not fully differentiate in the eye, although precursors of these T cells could be detected in the draining lymph nodes. Therefore, even though recipients with corneal allografts acquire donor-specific delayed hypersensitivity or cytotoxic T lymphocytes, the local microenvironment of the eye may suppress these immune responses at the local site.

T cells can be divided into two subpopulations on the basis of the expression of surface markers. T cells expressing CD4 molecules are mostly helper T cells that secrete cytokines and "help" the other immune cells to differentiate and function. T cells expressing CD8 molecules are mostly cytotoxic T cells that kill the target cells. Helper T cells can be classified into subsets that produce different sets of cytokines on activation. Th 1 cells produce IL-2, IFN-γ, and TNF-α but not IL-4 or IL-5, and predominate in cell-mediated immune responses, whereas Th 2 cells produce IL-4, IL-5, and IL-10, but not IL-2 or IFN-γ, and play a prominent role in humoral immunity. The present study has shown that the expression of IL-2 and IFN-γ is associated with graft rejection in corneal tissue. The results of immunohistochemical analysis indicate that the expression of these cytokines seems to have originated from the infiltrating cells in the rejected tissue. CD4 Th 1 cells and CD8 cytotoxic T cells are able to produce these cytokines; therefore, it is likely that these cells actually infiltrate into the donor graft tissue and cause graft destruction, which results in graft failure. He et al.10 reported that systemic anti-CD4 antibody administration to recipients promoted corneal allograft survival, whereas anti-CD8 antibody administration did not. They concluded that CD4-positive T cells, rather than CD8-positive T cells, play an important role in graft rejection. Therefore, infiltrating cells that secrete IL-2 and IFN-γ as detected in our studies may be CD4 T cells, which cause direct cytotoxicity or mediate a DH response in graft tissue.

The importance of the increases in IL-1α and TNF-α early after grafting is also noteworthy. Because these proinflammatory cytokines were detected in syngeneic and allogeneic grafted tissues at 1 week after grafting, these cytokines seemed to be secreted in response to the grafting procedure. Because IL-1 has been known to induce Langerhans cell migration into the central cornea,11 which is considered to be important for the recognition of alloantigens in grafted tissue, the increase in these proinflammatory cytokines early after grafting may con-
ttribute to graft rejection. In fact, Dana et al.\textsuperscript{12} have reported that the topical administration of IL-1 receptor antagonist after grafting promotes corneal allograft survival.

A recent study reported by Torres et al.\textsuperscript{13} found increased levels of proinflammatory cytokine mRNA up to 7 days postoperatively when both autografts and allografts showed mild to moderate opacity and revealed more expression of Th 1 cytokine mRNA than Th 2 cytokine mRNA in rejected allograft corneal tissue. Our results agree with this report and have demonstrated the increased expression of cytokine mRNA that was detected in their experiments, resulting in increased cytokine protein levels.

In summary, we have reported cytokine expression patterns after orthotopic corneal transplantation in grafted tissue. Our results showed that an increase in proinflammatory cytokine (IL-1α and TNF-α) expression was detected in syngeneic and allogeneic graft tissue and that the production of IL-2 and IFN-γ was detected only in rejected corneal allografts. These results suggest the importance of proinflammatory cytokines for the recognition of donor alloantigens in grafted tissue and the role of Th 1 cytokines as effectors of corneal allograft rejection in corneal tissue. We suggest that the inhibition of proinflammatory cytokines early after grafting or the inhibition of Th 1 cytokines may contribute to the prolongation of corneal allograft survival.

References

Decorin and Biglycan of Normal and Pathologic Human Corneas

James L. Funderburgh,\textsuperscript{1} Nathanael D. Hevelone,\textsuperscript{1} Mary R. Roth,\textsuperscript{1} Marthe L. Funderburgh,\textsuperscript{1} Merlyn R. Rodrigues,\textsuperscript{2} Verinder S. Nirankari,\textsuperscript{2} and Gary W. Conrad\textsuperscript{1}

\textbf{Purpose.} Corneas with scars and certain chronic pathologic conditions contain highly sulfated dermal sulfate, but little is known of the core proteins that carry these atypical glycosaminoglycans. In this study the proteoglycan proteins attached to dermatan sulfate in normal and pathologic human corneas were examined to identify primary genes involved in the pathobiology of corneal scarring.

\textbf{Methods.} Proteoglycans from human corneas with chronic edema, bullous keratopathy, and keratoconus and from normal corneas were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), quantitative immunoblotting, and immunohistochemistry with peptide antibodies to decorin and biglycan.

\textbf{Results.} Proteoglycans from pathologic corneas exhibit increased size heterogeneity and binding of the cationic dye alcian blue compared with those in normal corneas. Decorin and biglycan extracted from normal and diseased corneas exhibited similar molecular size distribution patterns. In approximately half of the pathologic corneas, the level of biglycan was elevated an average of seven times above normal, and decorin was elevated approximately three times above normal. The increases were associated with highly charged molecular forms of decorin and biglycan, indicating modification of the proteins with dermatan sulfate chains of increased sulfation. Immunostaining of corneal sections showed an