JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 29, Number 7, 2009

DOI: 10.1089/jir.2008.0116

Long-Term Kinetics of Cytokine Responses in Human Tears after Penetrating Keratoplasty

Mariann Fodor,[1] Péter Gogolák,[2] Éva Rajnavölgyi,[2] András Berta,[1] László Kardos,[3] László Módis,[1] and Andrea Facskó[1]

This was a kinetic study of inflammatory cytokine levels in postoperative tear samples from penetrating keratoplasty (PKP) patients with or without corneal rejection. In a prospective design, nonstimulated tears were collected from the affected eyes of 12 patients at regular intervals for 12–14 months following PKP. Nine patients retained clear grafts, whereas three suffered endothelial rejection of the corneal graft within 14 months. The concentrations of the cytokines IL-1β, IL-6, TNF-α, IL-8, IL-10, and IL-12p70 were measured via cytometric bead array technology. The postoperative concentrations of the cytokines in the tears varied among the patients, but exhibited similar alteration patterns in each eye tested. The concentrations of IL-6 and IL-8 were significantly higher ($P = 0.009$ and $P = 0.01$, respectively), whereas those of IL-10, TNF-α, and IL-12p70 were significantly lower ($P = 0.008$, $P = 0.006$, and $P = 0.0009$, respectively) in the tear samples from the patients with corneal rejection as compared with those with uncomplicated corneal grafts. The ratios IL-6/IL-10 and IL-8/IL-10 were significantly higher ($P = 0.0231$ and $P = 0.015$, respectively), and TNF-α/IL-10 was significantly lower ($P = 0.045$) throughout the examination period in the patients with endothelial rejection. The enhanced release of IL-6 and IL-8 into the tears of patients with corneal graft rejection concomitant with decreased concentrations of IL-10, TNF-α, and IL-12p70 may possibly serve as an indicator of the rejection process. However, due to the large variation in the cytokine concentrations, the observed changes in tear composition do not categorically predict the final graft outcome.

Introduction

Corneal graft rejection is one of the most significant complications of corneal transplantation (King and others 2000; Pleyer and others 2001; Xie and others 2003; Funding and others 2005). Despite the immunologically privileged nature of the cornea, immune-mediated graft rejection remains the major cause of unsuccessful human corneal allograft transplantation (Niederkorn and others 2004; Ritter and others 2007). The exact mechanisms involved in the initiation and effector functions of the immune system that mediate corneal allograft destruction remain unclear (Niederkorn and others 2004). The activity of immune cells causing graft rejection after penetrating keratoplasty (PKP) could be indirectly characterized by the determination of cytokine levels in the aqueous humor (AH) (Reinhard and others 2002) and it could be worthwhile to measure the cytokine levels in tears too. The importance and the role of various cytokines in different inflammatory diseases are well documented, but the levels and exact contributions of cytokines in human tears in the post-keratoplasty period are unknown (Torres and others 1996; van Gelderen and others 2000). The determination of different cytokines in noninvasively collected tears of patients with endothelial immune reactions may be the first approach to the identification of the cytokines involved in destruction of the graft endothelium.

Cytokines play a role in maintaining the integrity of the normal cornea (Torres and Kijlstra 2001). Because of the extreme complexity of the cytokine network, the simultaneous measurement of multiple cytokines in a single sample offers a feasible and efficient approach to compare the cytokine responses induced upon successful and failed keratoplasties (Chen and others 1999). A better understanding of

[1]Department of Ophthalmology and [2]Department of Immunology, Medical and Health Sciences Centre, Faculty of Medicine, University of Debrecen, Hungary.
[3]Medical Statistician, Head of Infection Control Services, Kenézy Hospital, Debrecen, Hungary.

cytokine secretion and functions upon graft rejection may allow improved therapeutic and preventive treatment modalities. Minor changes in the level of cytokine expressions may possibly mediate profound changes in the inflammatory response to alloantigenic stimuli (Kijlstra 1994). Instead of absolute concentrations, however, relative cytokine levels may be more valuable for the prediction of the local immunological response (Cook and others 2001; Uchino and others 2006a, 2006b; Sonoda and others 2006). Cytokine and chemokine expressions in the course of corneal transplant rejection have been studied at both mRNA (Torres and others 1996; Zhu and others 1999) and protein (Sano and others 1998; Yamagami and others 1998) levels in animal models, and at the protein level in the AH in human (van Gelderen and others 2000; Reinhard and others 2002; Funding and others 2005).

The detailed analysis of multiple cytokines was earlier hampered by the limited amount of tears available from a single eye. The microparticle-based flow cytometric bead array technology overcame this limitation as it allows the quantification of multiple cytokines in small samples (Chen and others 1999; Cook and others 2001; Tarnok and others 2003; Uchino and others 2006; Sonoda and others 2006; Uchino and others 2006; Malvitte and others 2007).

The present goal was a comparison of multiple cytokine patterns in tear samples collected from patients with or without corneal rejection following PKP. We are not aware of any previously published reports on this topic.

Materials and Methods

Patients and sample collection

In a prospective design, nonstimulated tears were collected from the affected eye of each of 11 patients at regular intervals for 1 year following PKP and in one transplant rejection case for 14 months. The mean age of the patients was 45.0 years (range 18–70 years, SD 14.2). Table 1 lists patient data and indications for PKP. None of the subjects were taking any medication that could interfere with tear production, and none suffered from any disease of known immunological origin. Following the tenets of the Helsinki Declaration, informed written consent was signed by all participants. All donor material was preserved in Optisol-GS (Bausch&Lomb, Rochester, NY) for at most 7 days. Routine medication (local corticosteroids and antibiotics) was applied for the first 12 months after corneal transplantation. Five patients received systemic anti-inflammatory therapy (i.v. or oral corticosteroid) to prepare them for rekeratoplasty or due to recipient vascularization.

Before tear collection, the anterior ocular status of each subject was carefully assessed; a slit-lamp under low illumination was used to avoid reflex tearing. Tear samples were collected in the morning before, and 1, 3, and 7 days after the operation, between 7.30 and 8.00 am, just before the first eye drops were instilled, and then at every ophthalmological control. Collection was nontraumatic, with capillary tubes, from the inferior meniscus, without topical anesthesia, during 2 min; the total volume of the collected tears was registered. The collected tear samples (overall 105) were frozen without centrifugation within 15 min and stored at –80°C until the cytokine measurements. Preliminary studies had demonstrated that centrifugation of the samples does not influence the cytokine concentrations. To avoid pipetting and dilution errors, collected tear samples of <4 µL were excluded. In some cases, dry eye did not allow tear collection. At the beginning of the rejection episode, sampling was performed before any additional medication.

Corneal endothelial rejection was diagnosed by the onset of an acute inflammatory episode combined with endothelial precipitates and/or stromal edema with increased central corneal thickness.

Cytokine measurements

The concentrations of six inflammatory cytokines (IL-8, IL-1β, IL-6, TNF-α, IL-10, and IL-12p70) were measured via

Table 1. Participating Patients and Indications for Penetrating Keratoplasty (PKP)

Patient	*Age (years)/sex*	*Cause of transplantation*	*Previous immune reactions*	*Days between transplantation and rejection*
1	24 M	Keratoconus	–	–
2	55 F	Herpes keratitis, corneal vascular leucoma, transplant rejection (second PKP)	+	–
3	70 F	Salzmann's nodular degeneration	–	–
4	18 F	Congenital hereditary endothelial dystrophy	–	–
5	59 F	Bullous keratopathy, transplant rejection (second PKP)	+	–
6	47 M	Bullous keratopathy	–	–
7	31 M	Keratoconus	–	–
8	22 F	Keratoconus	–	–
9	60 F	Salzmann's nodular degeneration	–	–
10	56 M	Herpes keratitis, transplant rejection (second PKP)	+	216
11	52 F	Haab-Dimmer dystrophy, recurrence of dystrophy (second PKP)	–	422
12	46 F	Chronic superficial keratitis (pannus)	–	83

the cytometric bead array (BD Biosciences Pharmigen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 15 μL of tear sample (in some cases diluted sample) or standard reagent was added to 15 μL of capture Ab-bead reagent. This mixture was incubated for 30 min and 15 μL of detector Ab–phycoerythrin conjugate was then added, followed by incubation for 2.5 h at room temperature and washing to remove any unbound reagent before data acquisition. Two-color flow cytometric analysis was performed with a FACS array cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Data were acquired and analyzed with the BD cytometric bead array software (FCAP Array 1.0.1 program). Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. During the preparation of the human cytokine standards, additional dilutions were prepared to achieve higher sensitivity. Assay sensitivities were 0.04 pg for TNF-α, IL-8, IL-1β, IL-12p70, and IL-6, and 0.02 pg for IL-10.

Statistical methods

Tear volumes, cytokine concentrations, and their ratios to that of IL-10 were compared by Wilcoxon's rank-sum test in tear samples of patients with rejection versus those with an uncomplicated engraftment. The group-specific overall concentrations of cytokines determined throughout the overall time course were calculated by using locally weighted regression analysis of outcomes against the day of follow-up in patients with and without rejection. The resulting Lowess curves were graphed on line charts. Statistical significance was set at $P < 0.05$.

Results

Twelve to fourteen months after the operation, nine patients presented clear grafts, whereas in three cases there was endothelial rejection of the corneal graft. All three rejected grafts had been predicted to be high-risk PKPs. The onset of immune rejection after transplantation was at 216, 422, or 83 days. Among the nine unrejected grafts, two had been expected to involve high-risk, and seven low-risk keratoplasty (Table 1). The tear sample volume collected from the patients with corneal rejection did not differ significantly from that collected from those with uncomplicated corneal grafts ($P = 0.096$).

The cytokine concentrations varied widely, but exhibited the same alteration pattern in each eye during the postoperative period. Early cytokine and chemokine responses induced by the transplantation were evident in all grafts. During the early postoperative phase (days 1–3), the levels of all tested cytokines rose, probably as a result of tissue injury rather than an allogeneic response (Fig. 1). The most pronounced increases were observed on day 1 for IL-6 (~25-fold) and IL-8 (nearly 5-fold), regardless of the occurrence of corneal rejection. The early response cytokine IL-1β, however, displayed a different alteration pattern: its initially low level increased slightly immediately after transplantation. In uncomplicated grafts, the level then declined slowly up to 6 months postoperatively, whereas in complicated grafts the early release was more pronounced, and before rejection a second peak was observed. The IL-8 concentration also increased before rejection, whereas the early IL-12p70 response was followed by a decline in both complicated and uncomplicated grafts. In uncomplicated corneal grafts, the similar biphasic IL-10 and TNF-α responses observed were presumably associated with the postoperative healing process. The slow IL-10 and TNF-α levels decreases gave way to a second cytokine release peak at about 1 year after PKP, although TNF-α was always detected at low levels, even upon rejection. By 12–14 months, the IL-1β, IL-6, and IL-8 concentrations in the tears from the uncomplicated graft cases had declined to the pretransplantation levels.

In the tears from the corneal rejection patients, the IL-6 and IL-8 concentrations increased ($P = 0.009$ and $P = 0.01$, respectively), whereas those of IL-10, TNF-α, and IL-12p70

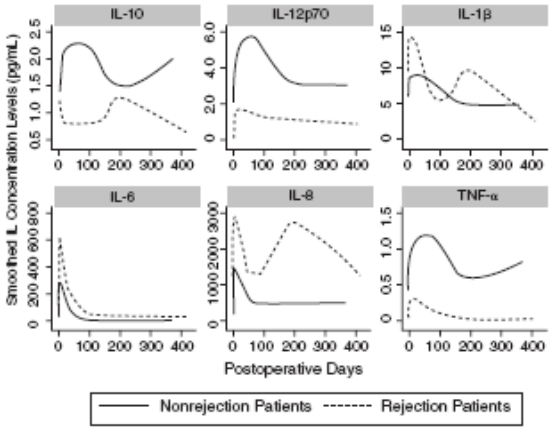

FIG. 1. Concentration (pg/mL) of cytokines in tears of patients with/without corneal rejection. Tear samples were collected at the indicated points of time and cytokine measurements were performed as described in the Materials and Methods. Data indicate smoothed group means obtained by locally weighted least squares regression.

decreased significantly ($P = 0.008$, $P = 0.006$, and $P = 0.0009$, respectively) relative to the uncomplicated corneal grafts, while the IL-1β concentration did not change significantly ($P = 0.383$).

As the balance of pro- and anti-inflammatory cytokines determines the inflammatory status of the eye, we calculated the ratios of the IL-6, TNF-α, and IL-8 concentrations to that of IL-10. In the patients with endothelial rejection, IL-6/IL-10 and IL-8/IL-10 were significantly higher ($P = 0.0231$ and $P = 0.015$, respectively), while TNF-α/IL-10 was significantly lower ($P = 0.045$) throughout the examination period than in those with uncomplicated grafts.

Discussion

Our results show that tears of patients who undergo corneal transplant rejection present significantly higher levels of IL-6 and IL-8 and lower levels of IL-10, TNF-α, and IL-12p70 than tears of transplanted patients without rejection. The constitutive release of six cytokines throughout a 12–14-month postoperative period was established. The levels of IL-1β, TNF-α, IL-10, and IL-12p70 remained constantly high after transplantation, even in uncomplicated grafts. The IL-6 and IL-8 concentrations dramatically increased and then rapidly declined 1–3 days after transplantation. These early cytokine responses could be attributed to the physical damage to the cornea and the presence of suture material.

Our study confirms the increase of IL-6 in AH of patients with corneal rejection (van Gelderen and others 2000; Funding and others 2005) by demonstrating a significantly increased IL-6 concentration in tears from patients with graft rejection relative to those without. In contrast, we demonstrate low IL-6 concentrations even in tears of patients with uncomplicated clear grafts, for which we suggest several possible explanations. IL-6 has the crucial functions of keeping the corneal button clear, stimulating collagen synthesis and supporting corneal wound healing, as well as being part of the endogenous anti-inflammatory system (Ventura and others 1997). Constant IL-6 and IL-10 expression has been observed postoperatively in rats receiving corneal allografts and for a shorter period in those receiving corneal autografts (Torres and others 1996). The discrepancies between this and other published reports could be due to differences in the indications for keratoplasty among the various subjects and the variability of the tissue samples (AH, cornea vs. tear). The sensitivities of the different methods (ELISA vs. CBA) could also hamper the comparison. Moreover, in vitro results on animal models may not be translated directly to the in vivo human situation (Klebe and others 2001).

In our human study, a second IL-1β and IL-8 concentration peak coincided with the onset of graft rejection. This second peak was likewise demonstrated in animal models of rejected cornea transplants where numerous cytokines were shown to be involved (King and others 2000). Interestingly, IL-1β was not detected in either normal or transplanted syngeneic or allogeneic corneal grafts in animal models, but TNF-α was profoundly enhanced following PKP (Zhu and others 1999). In our study, IL-1β reached background levels up to 6 months postoperatively in uncomplicated grafts. IL-1β has been considered a multifunctional cytokine in the cornea capable of initiating the inflammatory cascade and inducing corneal tissue damage, while contributing to tissue repair also (Torres and Kijlstra 2001). This could explain why no significant difference in the IL-1β levels was found in our tears from complicated and uncomplicated grafts. Although the IL-1β levels were not related to the transplantation outcome, it seems this cytokine is an active player in corneal rejection. Furthermore, IL-1β, IL-8, and TNF-α are also involved in neovascularization (Torres and Kijlstra 2001) and both TNF-α and IL-1β act as autocrine factors that can further enhance the expression of IL-6 and IL-8 (Ventura and others 1997). Similarly, our enhanced expression of the proinflammatory cytokines, IL-1β and TNF-α, at least partially provide the molecular basis of tissue infiltration observed in high-risk cornea transplantation (Yamagami and others 2005). The corneal TNF-α expression was found higher at both mRNA (Torres and others 1996) and protein levels in AH and serum from hosts with rejected corneal allografts (Pleyer and others 1997). In contrast, we found significantly decreased TNF-α levels in tears from patients with corneal rejection relative to those with uncomplicated corneal grafts. It has been suggested that TNF-α can induce apoptosis with corneal endothelial and epithelial cells susceptibility (Niederkorn and others 2004).

The reduced level of IL-10 in tears of patients with endothelial rejection could be an important attribute of the pathophysiology of transplant rejection. IL-10 has the potential to reduce the rejection incidence and prolong graft survival in animal models (Klebe and others 2001; Gong and others 2007; Chen and others 2007). Accordingly, we hypothesized that increased levels of tear IL-10 in eyes with clear corneal grafts could be an indicator of graft tolerance, whereas during rejection IL-10 can act as an inhibitory factor for T-helper Type 1 responses. The IL-10 concentration was significantly lower in tears of patients with rejection compared to those with nonrejection. IL-10 decreases the expression of MHC class II in monocytes/macrophages, thus interfering with their antigen-presenting function. IL-10 also modulates monocytes by suppressing the production of other proinflammatory cytokines, TNF-α, IL-1β, and IL-8 (Dallman 1993), and is released after apoptosis induction in activated T cells (King and others 2000). The reduced level of IL-10 in AQ2 the tears of patients with rejection results in a trend toward increased ratios of IL-6/IL-10 and IL-8/IL-10, and decreased TNF-α/IL-10. Disruption of the balance of pro- and anti-inflammatory cytokines may lead to transplant rejection and decreased corneal graft tolerance.

The induction of immunity to graft antigens in the draining lymph nodes after cornea transplantation occurs via an IL-12 and INF-γ-dependent mechanism (Liu and others 2001). A significant up-regulation of IL-12 mRNA was observed in rejected corneal allografts in rats (King and AQ3 others 2000). The local delivery of IL-12p40 results in partial inhibition of activated T-cell infiltration and the release of Th1 cytokines, both playing critical roles in corneal allograft rejection, but not sufficient to prevent rejection (Torres and others 1996; Ritter and others 2007). Our results reveal significant decrease in IL-12p70 in tears of patients with corneal rejection compared to uncomplicated grafts along with the results by Klebe et al. (2005).

The present study contains few limitations: constrained number of patients involved in the tear sampling; few tear samples having cytokine concentrations near the detection limit; treatments in the different patient groups being not completely similar (i.v. or oral steroid was mainly used in the graft rejection group). Our stratified statistical data analysis

revealed that the systemic administration of steroids did not exert a confounding effect on our results. We hoped to identify a cytokine that could be used as a marker of transplant rejection in human tears; however, further investigations are needed to identify it. This study is a first step toward establishing immunological analysis of tears from patients undergoing keratoplasty that could be used as a predictor of clinical status and need for preventive therapy.

Acknowledgment

This work was supported by the Hungarian Research Fund (OTKA TO 383483).

References

Chen B, Kapturczak MH, Joseph R, Agarwal M, Navarro D, Santamaria P, Perez R, Del Castillo D, Aljama P. 2007. Adeno-associated viral vector-mediated interleukin-10 prolongs allograft survival in a rat kidney transplantation model. Am J Transplant 7(5):1112–1120.

Chen R, Lowe L, Wilson JD, Crowther E, Tzoggai K, Bishop JE, Varro R. 1999. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. Clin Chem 45(9):1693–1694.

Cook EB, Stahl JL, Lowe L, Chen R, Morgan E, Wilson J, Varro R, Chan A, Graziano FM, Barney NP. 2001. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. J Immunol Methods 254:109–118.

Dallman MJ. 1993. Cytokines as mediators of organ graft rejection and tolerance. Curr Opin Immunol 5(5):788–793.

Dana MR, Dai R, Zhu S, Yamada J, Streilein JW. 1998. Interleukin-1 receptor antagonist suppresses Langerhans cell activity and promotes ocular immune privilege. Invest Ophthalmol Vis Sci 39:70–77.

Funding M, Vorum H, Nexø E, Moestrup SK, Ehlers N, Møller HJ. 2005. Soluble CD163 and interleukin-6 are increased in aqueous humour from patients with endothelial rejection of corneal grafts. Acta Ophthalmol Scand 83:234–239.

van Gelderen BE, van der Lelij A, Peek R, Broersma L, Treffers WF, Ruijter JM, van der Gaag R. 2000. Cytokines in aqueous humor and serum before and after corneal transplantation and during rejection. Ophthalmic Res 32:157–164.

Gong N, Pleyer U, Volk HD, Ritter T. 2007. Effects of local and systemic viral interleukin-10 gene transfer on corneal allograft survival. Gene Ther 14:484–490.

Kijlstra A. 1996. The role of cytokines in ocular inflammation. Br J Ophthalmol 78:865–866.

King WJ, Comer RM, Hodder T, Larkin DFP, George AJT. 2000. Cytokine and chemokine expression kinetics after corneal transplantation. Transplantation 70:1225–1233.

Klebe S, Coster DJ, Sykes PJ, Swinbourne S, Hallsworth P, Schoenfeld JF. 2003. Prolongation of sheep corneal allograft survival by transfer of the gene encoding ovine IL-12-p40 but not IL-4 to donor corneal endothelium. J Immunol 171:2219–2226.

Klebe S, Sykes PJ, Coster DJ, Krishnan R, Williams KA. 2001. Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. Transplantation 71:1224–1230.

Liu Y, Dana MR, Tewari V, Taylor AW. 2001. Immune response to intragraft antigen in draining lymph nodes after corneal transplantation is mediated by interleukin-12. J Interferon Cytokine Res 21:813–819.

Malvitte L, Montange T, Vejux A, Baudouin C, Bron AM, Creuzot-Garcher C, Lizard G. 2007. Measurement of inflammatory cytokines by multicytokine assay in tears of patients with glaucoma topically treated with chronic drugs. Br J Ophthalmol 91:29–32.

Niederkorn JY, Mayhew E, Mellon J, Hedge S. 2004. Role of tumor necrosis factor receptor expression in anterior chamber-associated immune deviation (ACAID) and corneal allograft survival. Invest Ophthalmol Vis Sci 45:2874–2881.

Pleyer U, Dannowski H, Volk H-D, Ritter T. 2001. Corneal allograft rejection: current understanding. Ophthalmologica 215: 254–262.

Pleyer U, Milani JK, Ruckert D, Rieck P, Mondino BJ. 1997. Determination of serum tumor necrosis factor alpha in corneal allografts. Ocul Immunol Inflam 5:149–155.

Reinhard T, Böcking A, Poenjarski N, Sundmacher R. 2002. Immune cells in the anterior chamber of patients with immune reactions after penetrating keratoplasty. Cornea 21(1):56–61.

Ritter T, Yang J, Dannowski H, Vogt K, Volk HD, Pleyer U. 2007. Effects of interleukin-12p40 gene transfer on rat corneal allograft survival. Transplant Immunol 18:101–107.

Sano Y, Osawa H, Sotozono C, Kinoshita S. 1998. Cytokine expression during orthotopic corneal allograft rejection in mice. Invest Ophthalmol Vis Sci 39:1953–1957.

Soneda S, Uchino E, Nakao K, Sakamoto T. 2006. Inflammatory cytokine of basal and reflex tears analysed by multicytokine assay. Br J Ophthalmol 90(1):120–122.

Tarnok A, Hambsch J, Chen R, Varro R. 2003. Cytometric bead array to measure six cytokines in twenty-five microliters of serum. Clin Chem 49:1000–1002.

Torres PE, De Vos AF, van der Gaag R, Martins B, Kijlstra A. 1996. Cytokine mRNA expression during experimental corneal allograft rejection. Exp Eye Res 62:452–461.

Torres PF, Kijlstra A. 2001. The role of cytokines in corneal immunopathology. Ocular Immunol Inflamm 9:9–26.

Uchino E, Soneda S, Kinukawa N, Sakamoto T. 2006a. Alteration pattern of tear cytokines during the course of a day: Diurnal rhythm analyzed by multicytokine assay. Cytokine 33:36–40.

Uchino E, Soneda S, Nakao K, Sakamoto T. 2006b. Alteration of tear cytokine balance by eye closure: analysis by multicytokine assay. Graefe's Arch Clin Exp Ophthalmol 244:747–749.

Vestrum ACS, Engelmann K, Dahinden C, Böhnke M. 1997. Endotoxin modulates the autocrine function of organ cultured donor corneas and increase the incidence of endothelial cell death. Br J Ophthalmol 81:1092–1098.

Xie L, Shi W, Cao P. 2003. Roles of tumor necrosis factor-related apoptosis inducing ligand in corneal transplantation. Transplantation 76:1556–1559.

Yamagami S, Hamrah P, Zhang Q, Liu Y, Haq S, Dana MR. 2005. Early ocular chemokine gene expression and leukocyte infiltration after high-risk corneal transplantation. Mol Vis 11: 622–940.

Yamagami S, Kawashima H, Endo H, Tsuru T, Shibui H, Kagawa Y, Hori J, Yamagami H, Inobe M. 1998. Cytokine profiles of aqueous humor and graft in orthotopic mouse corneal transplantation. Transplantation 66:1534–1540.

Zhu S, Dekaris I, Darcicor G, Dana R. 1999. Early expression of proinflammatory cytokines interleukin-1 and tumor necrosis factor-a after corneal transplantation. J Interferon Cytokine Res 19:661–668.

Address reprint requests or correspondence to:
Mariann Fodor, MD
Department of Ophthalmology
University of Debrecen
Nagyerdei krt. 98
H-4012 Debrecen
Hungary

Tel/Fax: + 36 52 415836
E-Mail: infodoc@dote.hu

Received 4 December 2008/Accepted 7 January 2009

AQ1: Please approve the change made in the year from "2006" to "2006a, 2006b" as in the reference list
AQ2: Please approve the change made in the author name from "Kind" to "King" as in the reference list or else provide complete reference details for Kind and others 2003 in the reference list.
AQ3: Please approve the change made in the author name from "Kind" to "King" as in the reference list or else provide complete reference details for Kind and others 2003 in the reference list.
AQ4: Please provide in-text citation for Dana and others 1998, as other references cited in the text.