Prophylactic Effect of IL-10 Gene Transfer on Induced Autoimmune Dacryoadenitis

Zejin Zhu,[1] *Douglas Stevenson,*[1] *Joel E. Schechter,*[2] *Austin K. Mircheff,*[1,3,4] *Thomas Ritter,*[5] *Laurie Labree,*[6] *and Melvin D. Trousdale*[1,3]

Purpose. To evaluate the effect of viral IL-10 on the lacrimal gland immunopathologic response in the ocular surface disease, induced autoimmune dacryoadenitis.

Methods. Disease was induced in rabbits by injecting inferior lacrimal glands with peripheral blood lymphocytes activated by 5 days of coculture with autologous acinar cells in a mixed-cell reaction. In the treated group, an adenoviral vector carrying the vIL-10 gene was concurrently injected with activated lymphocytes. Tears were collected periodically for quantitation of IL-10 by ELISA. Two weeks after disease induction, tear production, tear film breakup time, and rose bengal staining score were determined. Sectioned glands were immunostained for expression of CD4, CD8, rabbit thymic lymphocyte antigen (RTLA), CD18 and major histocompatibility complex class II.

Results. The titer of vIL-10 in tears was at its maximum on day 3, started to decline by day 7, and was undetectable by day 14. In the diseased group, the tear production rate and tear film breakup time were significantly decreased, and rose bengal staining was significantly increased. Diseased glands had immune cell infiltrates containing $CD4^+$, $RTLA^+$, and $CD18^+$ cells, and major histocompatibility complex class II expression was increased. These changes were significantly ameliorated by expression of vIL-10.

Conclusions. In vivo transduction of the lacrimal gland with AdvIL-10 resulted in the transient appearance of vIL-10 in tears. The presence of vIL-10 partially suppressed the appearance of Sjögren-syndrome–like features of reduced tear production, accelerated tear breakup, ocular surface disease, and immunopathologic response. Anti-inflammatory cytokine gene expression may offer a therapeutic modality for the treatment of autoimmune dacryoadenitis, once suitable vectors become available. (*Invest Ophthalmol Vis Sci.* 2004;45:1375–1381) DOI:10.1167/iovs.03-0755

Sjögren's syndrome (SjS), one of the most common autoimmune diseases, primarily affects exocrine glands. It causes persistent dysfunction of the salivary and lacrimal glands and is associated with the objective findings of xerostomia and keratoconjunctivitis sicca.[1] A combination of immunologic, genetic, hormonal, and environmental factors may play a role in the development of autoimmunity in the lacrimal gland.[2,3] Mononuclear cell infiltration proximal to epithelial cells of the exocrine glands is the typical pathologic finding. The infiltrates consist primarily of $CD4^+$ cells. An inflammatory, or Th-1 type, cytokine profile generally has been thought to be characteristic of SjS. There is evidence that the infiltrates produce proinflammatory cytokines, including interleukin (IL)-1, -6, -12, and -18; interferon (IFN)-γ; and tumor necrosis factor (TNF)-α.[4–8] However, in addition to the regulatory cytokines IL-10 and transforming growth factor (TGF)-β1, the Th-2 cytokine IL-4 is detected in some samples,[9–10] where it has been suggested to be associated with B-cell proliferation.[11] Moreover, there is evidence that the epithelial cells themselves can significantly influence the overall cytokine profile and the biological consequences of mononuclear cell infiltration.[9,12]

Interleukin-10 is a cytokine originally identified as a "cytokine synthesis inhibitory factor" because of its ability to suppress cytokine synthesis in Th1 cells.[13] In vitro and in vivo studies show that IL-10 inhibits macrophage/monocyte-dependent antigen presentation by downregulating the expression of major histocompatibility complex (MHC) class II, costimulatory molecules (CD40, CD80, and CD86), and intercellular adhesion molecule (ICAM)-1. In addition, IL-10 strongly inhibits production of cytokines (such as Th1 differentiating cytokine, IL-12) and proliferation of $CD4^+$ T cells, thereby inducing and maintaining T-cell nonresponsiveness or anergy.[14,15] However, IL-10 also has immunostimulatory properties, especially on $CD8^+$ T cells and B cells.[14] These pleiotropic effects may explain the discrepancy observed after IL-10 treatment in different in vivo experimental models. Human IL-10 and the vIL-10 encoded by the Epstein-Barr virus have an 84% sequence homology, with most differences occurring in the N-terminal 20 amino acids. The vIL-10 mimics several immunosuppressive activities of human IL-10; however, unlike human IL-10, vIL-10 does not enhance class II MHC expression on mouse B cells,[16] nor does it effectively costimulate mouse thymocyte or mast cell proliferation.[17]

Interleukin-10 is regarded as a potential therapeutic agent for inflammatory diseases involving Th1 responses because of its ability to downregulate several major functions of Th1 cells and macrophages. Currently, recombinant human IL-10 is being tested in such human autoimmune diseases as rheumatoid arthritis, Crohn's disease, multiple sclerosis, and psoriasis.[18,19] In a previous study, using an adenovirus construct capable of inducing transient vIL-10 gene expression, we demonstrated that lacrimal gland epithelial cells (LGECs) can be transduced in vitro and that detectable, functional vIL-10 suppresses the ability of these cells to activate lymphocytes in autologous mixed-cell reactions.[20] More recently, we reported that progressive lacrimal gland dysfunction and ocular surface disease— characterized by reduced tear production, reduced tear stability, and abnormal staining of the cornea surface, and an increased number of infiltrating $CD4^+$, $RTLA^+$, and $CD18^+$ cells— developed in animals whose lacrimal glands were injected with LGEC-stimulated lymphocytes.[21] These important

From the [1]Doheny Eye Institute and the Departments of [2]Cell and Neurobiology, [3]Ophthalmology, [4]Physiology and Biophysics, and [6]Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California; and the [5]Institute of Medical Immunology, Humboldt University, Berlin, Germany.

Supported in part by National Eye Institute Grants EY12689, EY05801, EY10550, and EY03040 and by grants from Research to Prevent Blindness and Allergan Corp. (Irvine, California).

Submitted for publication July 17, 2003; revised September 10, and November 12, 2003; accepted November 26, 2003.

Disclosure: **Z. Zhu**, None; **D. Stevenson**, None; **J.E. Schechter**, None; **A.K. Mircheff**, None; **T. Ritter**, None; **L. Labree**, None; **M.D. Trousdale**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Melvin D. Trousdale, Doheny Eye Institute, 1450 San Pablo Street, Los Angeles, CA 90033; trousdal@hsc.usc.edu.

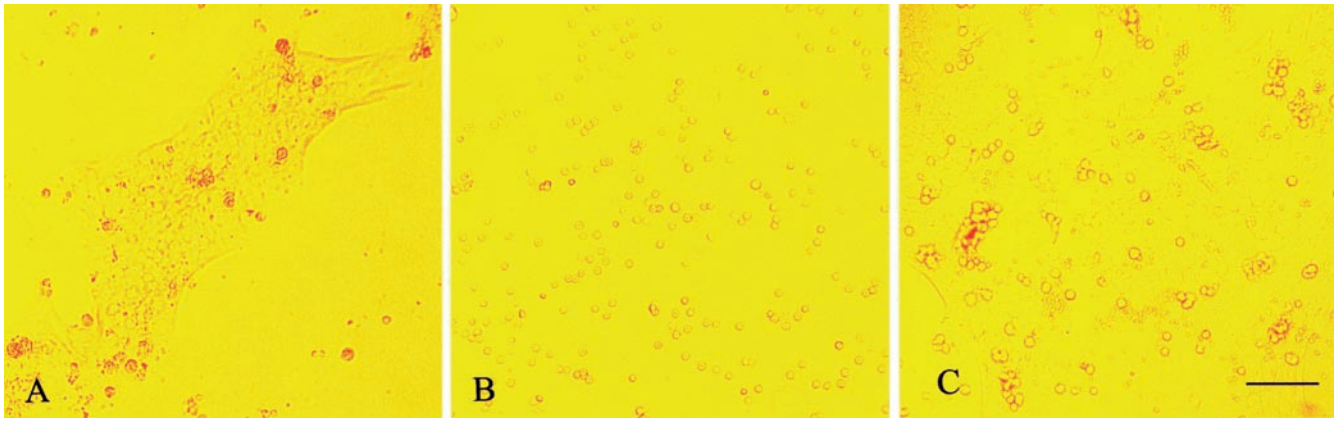

FIGURE 1. Mixed-cell reaction was composed of (**A**) pLGECs cultured for 2 days and then gamma irradiated; (**B**) PBLs cultured alone for 2 days, appearing as small round cells; (**C**) PBLs were cocultured with the gamma-irradiated pLGECs for 5 days. Activated lymphocytes become enlarged and more irregular shaped than unstimulated lymphocytes. The activated lymphocytes can be collected and injected directly into the donor animal's remaining lacrimal gland and autoimmune dacryoadenitis appears in 14 days. Bar, 100 μm.

features of severe dry eye and SjS were used in the present study to evaluate the effect of adenovirus-mediated gene transfer and expression of vIL-10 on the lacrimal gland immunopathologic response and ocular surface disease resulting from induced autoimmune dacryoadenitis.

METHODS

Animals, Surgical Procedures, and Clinical Assessments

Female New Zealand White rabbits (3.5–4 kg) were obtained from Irish Farms (Norco, CA). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were maintained in a facility fully accredited by the American Association for Laboratory Animal Science. Before experimentation, animals were examined and tested by two investigators. The clinical assessment, including slit lamp biomicroscopy, Schirmer's tear test, tear breakup time (BUT), and rose bengal staining, were performed at time 0 and 2 weeks after injection, using previously published protocols.[21] Schirmer test paper strips were purchased from Chauvin Pharmaceuticals Ltd. (Romford, UK), fluorescein from Alcon Laboratories Inc. (Fort Worth, TX), and rose bengal strips from Akorn Inc. (Abita Springs, LA). There were five animals in each study group.

After anesthesia, the left lacrimal gland was surgically removed from each rabbit for the preparation of the purified (p)LGECs, as previously described.[21] These eyes received topical applications of bacitracin-neomycin-polymixin veterinary ophthalmic ointment (Pharmaderm; Altana, Inc., Melville, NY). Intramuscular injections of buprenorphine HCl (20 μg/kg twice daily; Reckitt & Colman, Hull, UK) were administered to the rabbits for the first two postoperative days. Peripheral blood was also obtained for lymphocyte preparation for the mixed-cell reaction.

Vectors

A replication-defective adenovirus construct carrying the Epstein-Barr virus IL-10 gene (AdvIL-10) was used.[20] Briefly, the cDNA for vIL-10 was subcloned into the pACCMV vector to generate recombinant replication-deficient virus. Adenovirus plaques were selected and propagated in strain 293 cells. A cesium-chloride–banded vector was recovered and dialyzed, then stored in aliquots at −80°C after the addition of 10% glycerol.

Tear Collection and ELISA for Measuring vIL-10

Basal tears were collected by lightly touching a 5-μL micropipet to the lacrimal lake (inferior conjunctiva near the lower posterior lid margin and the nictitating membrane). After low-speed centrifugation, the supernatant was collected, diluted 1:2 in phosphate-buffered saline, and supplemented with a cocktail of protease inhibitors to yield the following final concentrations: *N*(α)-*p*-tosyl-L-arginine methyl ester (TAME) 10 μg/mL (Sigma-Aldrich, St. Louis, MO), *N*(α)-*p*-tosyl-L-lysin chloromethyl ketone (TLCK) 10 μg/mL (Sigma-Aldrich), and leupeptin 1 μg/mL (Sigma-Aldrich). The treated tear samples were stored at −70°C. vIL-10 concentrations were quantified with a viral IL-10 ELISA kit (BioSource Europe, SA, Nivelles, Belgium) according to the manufacturer's recommended protocol, and the concentrations were determined from the standard curve. Tears were collected at time 0 and 3, 7, and 14 days after inoculation.

Cell Culture and Immunocytochemical Reagents

Hepato-Stem Hepatocyte Defined Medium was purchased from BD Biosciences (Bedford, MA). Ham's F12 medium and antibiotic-antimycotic mixture were purchased from Invitrogen-Gibco products (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Omega Scientific (Tarzana, CA). Bovine serum albumin, soybean trypsin inhibitor, and linoleic acid were purchased from Sigma-Aldrich. Tritiated thymidine was purchased from DuPont NEN Research Products (Wilmington, DE). Monoclonal antibodies to rabbit CD4, CD8, and CD18 were purchased from Spring Valley Laboratories (Woodbine, MD). Goat antiserum to RTLA was obtained from Cedar Lane Laboratories (Hornby, Ontario, Canada). Biotin labeled goat anti-mouse IgG Fc and donkey anti-goat IgG were purchased from Chemicon International (Temecula, CA). An avidin-biotin complex kit (Vectastain Elite ABC) was obtained from Vector Laboratories (Burlingame, CA).

Autologous Mixed-cell reaction and Induction of Autoimmune Dacryoadenitis

Descriptions of lacrimal gland excision, acinar cell purification, and mixed-cell reaction procedures have been published.[20] Purified pLGECs and peripheral blood lymphocytes (PBLs) were isolated and cultured separately for 2 days (Figs. 1A, 1B). A mixed-cell reaction was performed in 12-well plates, using an equal number (1×10^6/mL) of autologous PBLs and 2500-rad γ-irradiated pLGECs, then cocultured for 5 days. The cells were also cultured in 96-well plates under the same conditions, but using 1×10^5 cells of each type to monitor PBL stimulation. The cells were treated with [^{3}H]thymidine after 4 days in coculture and harvested 24 hours later with a commercial sample harvester (model 290 PHD; Brandel, Gaithersburg, MD). A beta scintillation counter (model LS 6000IC; Beckman Instruments Inc., Fullerton, CA) was used to measure [^{3}H]thymidine incorporation. A minimum of six wells was counted to obtain representative data.

TABLE 1. ELISA of vIL-10 Transgene Expression in Tears after Adenovirus-Mediated Transduction

Time after Injection (d)	IL-10 Concentration (pg/mL)	Median (range)
0	0, 0, 0, 0, 0*	0 (0–0)
3	65, 39, 103, 94, 77	77 (39–103)
7	28, 0, 128, 43, 35	35 (0–128)
14	0, 0, 0, 0, 0	0 (0–0)

For each time point, $n = 5$. AdvIL-10 was mixed with activated lymphocytes from the mixed cell reaction before the lacrimal gland was injected.

* IL-10 undetectable.

The stimulation index is the ratio of the average scintillation count of the mixed-cell reaction group to that of PBL control. Lymphocytes from mixed-cell reactions with stimulation indices greater than two were considered to contain activated lymphocytes. Activated lymphocytes from the parallel cultures were centrifuged, suspended in fetal bovine serum–free medium, and then injected into the central region of the inferior lacrimal glands of the right eyes of the respective donor rabbit, using a 25-gauge butterfly needle on a tuberculin syringe. To avoid tissue damage caused by rapid volume expansion, the microvolume delivery system was set to deliver 10 μL at 10-second intervals (10 μL $\times$ 20 repetitions = 200 μL). Rabbits receiving activated lymphocytes are hereafter referred to as being in the induced dacryoadenitis (ID) group. Those ID animals that received AdvIL-10 (1×10^8 pfu) are hereafter referred to as ID/AdvIL-10–treated animals.

Tissue Collection and Histopathology

Rabbits were killed by injection of pentobarbital sodium (56 mg/kg) 14 days after inoculation. The inferior lacrimal glands of the right eyes were removed from each animal and dissected longitudinally into two parts. One part was fixed in 10% formalin and embedded in paraffin, and the other part was embedded in optimal cutting temperature (OCT) compound (Miles, Inc., Elkhart, IN), frozen, and stored at −70°C until histologic examination. Throughout these studies, a standardized protocol was followed to maintain continuity from experiment to experiment.[21] Lymphocytes were always inoculated into the central region of the gland using the method described earlier. During tissue collection, a known orientation (i.e., nasal to temporal) was maintained, and serial sections from the central region of the gland were mounted, stained, and examined in a consistent manner. Cryosections were used, and immunohistologic staining was performed according to published protocols.[21] Hematoxylin counterstain was applied before mounting. Cells that stained positive exhibited an intense brown color and were readily distinguished from the blue background. The entire section was scanned and analyzed with an automated cellular imaging system (ACIS; ChromaVision Medical Systems, Inc., San Juan Capistrano, CA). The ACIS, a proprietary, color-based imaging technology with automated microscopy, provides quantitative data, including the percentage of cells positive, intensity scoring, and area measurement. The ACIS is designed to measure the total area occupied by all cells in a scanned section and also the percentage of that area that is occupied by cells expressing a specific cell marker (e.g., CD4). Four sections per gland and five glands per study group (20 sections) were scanned and analyzed for each marker. The results are presented as the mean percentage positive for each expressed marker (i.e., area stained for specific antigen divided by total area of tissue scanned $\times$ 100 = percentage positive).

Immunohistochemical analyses of data for expression of each marker (i.e., CD4, CD8, RTLA, CD18, or MHC-II) are presented as mean percentage positive from four sections per sample (i.e., area stained for specific antigen divided by total area of tissue scanned $\times$ 100 = percentage positive).

Statistics

Data collected from clinical analysis and the ACIS (ChromaVision) were subjected to paired *t*-test or signed rank sum test and analysis of variance (ANOVA). Multiple comparison *t*-tests were calculated when ANOVA $P \leq 0.05$. Statistical significance was adjusted for the number of pair-wise comparisons with a Bonferroni correction.

RESULTS

Detection of vIL-10 in Tears

The appearance and duration of vIL-10 in tears after the injection of activated lymphocytes and the adenovirus vector carrying the vIL-10 gene were monitored by ELISA (Table 1). On day 0 before injection, tear samples from all animals were negative for IL-10. Three days later, vIL-10 concentrations ranged from 35 to 103 pg/mL in tears of treated animals. By day 7, vIL-10 titers declined in four of five animals, whereas the titer in one animal increased slightly from 103 to 128 pg/mL. By day 14, IL-10 was undetectable in tears.

Activation of Lymphocytes and Clinical Assessment

The activation of PBLs was demonstrated by the incorporation of [^{3}H]thymidine in the mixed-cell reactions with autologous γ-irradiated pLGECs. [^{3}H]thymidine incorporation in stimulated PBLs increased from 2.9- to 5.8-fold, compared with results obtained using PBLs only (data not shown). Microscopically, the activated PBLs appeared enlarged, irregularly shaped, and clustered (Fig. 1 C) compared with nonactivated PBLs (Fig. 1B).

The clinical assessments are presented in Figures 2, 3, and 4. At time 0, the Schirmer test scores were 8.0 $\pm$ 0.9 and 8.3 $\pm$ 1.1 mm in the ID and ID/AdvIL-10–treated groups, respectively ($P = 0.70$). At 2 weeks, the Schirmer test score in the ID group decreased significantly ($P < 0.05$) to a final value of 5.0 $\pm$ 1.1 mm. In contrast, the score in the ID/AdvIL-10–treated group remained virtually unchanged at 7.9 $\pm$ 1.5 mm. The difference between the Schirmer scores of the ID and ID/AdvIL-10–treated groups was statistically significant ($P < 0.05$).

The tear BUTs at time 0 were 21 $\pm$ 2.9 and 18.6 $\pm$ 2.6 seconds in the ID and ID/AdvIL-10–treated groups, respectively ($P = 0.21$). At 2 weeks, scores in the ID group were decreased almost 50%, to 12.1 $\pm$ 2.1 seconds. In contrast, the 2-week BUT in the ID/AdvIL-10–treated group (18.6 $\pm$ 2.6

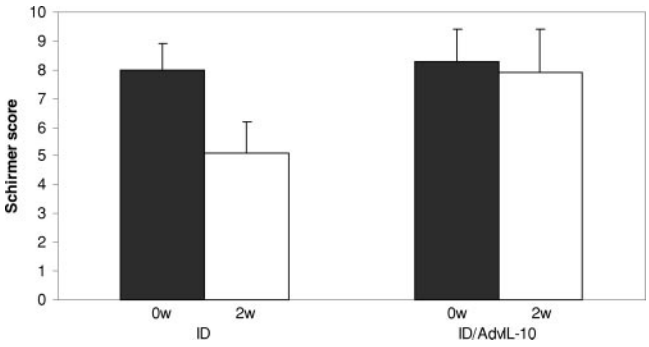

FIGURE 2. Basal tear production. The Schirmer test was performed on five rabbits (0 w), and dacryoadenitis was induced in these animals with lymphocytes activated in a mixed-cell reaction (i.e., designated ID group). In the ID/AdvIL-10–treated group, AdvIL-10 (1×10^8 pfu) was injected concurrently into the lacrimal gland of ID animals. Two weeks later (2 w), the Schirmer test was repeated in both groups. Tear production was significantly reduced at 2 weeks in the ID group compared with the ID/AdvIL-10 group ($P < 0.05$).

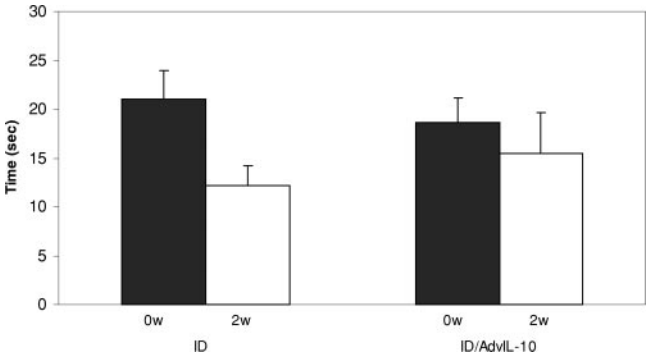

FIGURE 3. Tear BUT was determined before surgery (0 w) and after 2 weeks (2 w). Designations of ID and ID/AdvIL-10–treated groups are the same as described in Figure 2. At 2 weeks, tear BUT was significantly decreased in the ID group compared with the ID/AdvIL-10 group ($P < 0.05$).

seconds) was not significantly different from the time 0 value. The difference between the tear BUTs of the ID and ID/AdvIL-10–treated groups at 2 weeks was statistically significant ($P < 0.05$).

The rose bengal scores were 0.4 ± 0.6 and 0.2 ± 0.5, respectively, for the ID and ID/AdvIL-10–treated groups at time 0. At 2 weeks, the scores had increased to 2.6 ± 0.9 in the ID group and 1.6 ± 0.6 in the ID/AdvIL-10–treated group. The difference between the two groups was not statistically significant ($P = 0.13$).

Classification of Infiltrating Immune Cells

Microscopic examination revealed focal immune cell infiltrates in glands from both ID and ID/AdvIL-10–treated animals (Figs. 5A, 5B, respectively), but not in glands from normal animals. However, the infiltrates in the glands of ID/AdvIL-10–treated animals appeared smaller and fewer than those in the glands of the ID animals, as was confirmed by image analysis. Both ID and ID/AdvIL-10–treated glands contained numerous cells strongly positive for $CD4^+$, localized in dense foci around ducts and venules (Figs. 5C, 5D, respectively). As noted previously, some of the venules exhibited a high endothelial phenotype. $CD8^+$ cells were evident in perivenular areas and between acini and were occasionally seen within venules. They appeared much more abundant in ID/AdvIL-10–treated glands than in ID glands (Figs. 5E, 5F, respectively). In both groups, cells intensely positive for RTLA were present in dense foci associated with venules and to a lesser extent around ducts (Figs. 5G, 5H, respectively). $RTLA^+$ cells were also abundant between acini. Intensely positive $CD18^+$ cells were also evident in perivenular foci and between acini of both ID and ID/AdvIL-10–treated tissues, but they were less abundant in the ID/AdvIL-10–treated tissues (Figs. 5I, 5J, respectively).

Quantitative image analysis with the ACIS system confirmed that ID glands contained significantly more $CD4^+$, $RTLA^+$, and $CD18^+$ cells than normal or ID/AdvIL-10–treated glands and that the number of $CD8^+$ cells was significantly greater in the ID/AdvIL-10–treated group (Table 2). MHC-II expression by interstitial and ductal epithelial cells was significantly more frequent in the ID group than in normal glands; MHC-II expression in the ID/AdvIL-10–treated group (Fig. 5L) was significantly less than in the ID group (Fig. 5K) and was not significantly different from that in the normal group. Statistical pairwise comparisons of these results are shown in Table 2.

DISCUSSION

IL-10 is considered an immunoregulatory cytokine because of its inhibitory effects on the expression of a large spectrum of proinflammatory cytokines (such as IL-1α, -1β, -3, -6, -8; TNFα; granulocyte colony-stimulating factor [G-CSF], and granulocyte-macrophage colony-stimulating factor [GM-CSF]), chemokines (including IL-8 and macrophage inflammatory protein [MIP]-α), MHC-II molecules, costimulatory molecules (such as CD40, CD80, and ICAM-1), and other inflammatory mediators (free oxygen radicals and nitric oxide).[8,13,22,23] Numerous reports emphasize the role that IL-10 plays in the downregulation of ocular inflammation and in the immune privilege of the eye.[24–26] In contrast to cellular IL-10, Epstein Barr virus-encoded vIL-10 homologue shows only immunosuppressive properties, and it lacks cellular IL-10's stimulatory effects on natural killer cells and cytotoxic T cells.[16,17]

An adenovirus vector carrying the vIL-10 gene has been reported to suppress inflammation successfully in several animal models.[26–33] De Kozak et al.[26] reported that the subconjunctival injection of AdvIL-10 significantly decreases the severity of experimental autoimmune uveoretinitis without affecting the systemic immune response. Their findings are consistent with previous results observed in IL-10–treated mice, indicating that IL-10 can suppress the Th1-type response without promoting a Th2 shift in the experimental autoimmune uveoretinitis system. Klebe et al.[32] also demonstrated that ex vivo transduction of corneas before transplantation with an adenovirus encoding for IL-10 prolonged survival in a sheep keratoplasty model. In a previous study, we demonstrated that AdvIL-10 gene transduction significantly inhibits PBL activation in an autologous acinar cell–PBL mixed-cell reaction.[20] These findings demonstrate the applicability and the promising beneficial effects of AdvIL-10 gene transduction in suppressing inflammation.

In our previous in vivo gene transduction study, we reported that a single injection of adenoviral vector carrying a green fluorescent protein (GFP) marker gene into the rabbit lacrimal gland resulted in GFP expression for up to 2 weeks in various types of cells in the gland, including acinar, ductal, and interstitial cells.[33] In the same study, we also reported the effect of the adenoviral vector on normal lacrimal glands. Briefly, the number of $CD4^+$ or $CD8^+$ cells in AdGFP-injected glands was not significantly different from that in normal glands. The number of $RTLA^+$ and $CD18^+$ cells was increased, compared with those cell types in normal glands, but they remained significantly less than in ID tissues and in ID glands that had been concurrently injected with an adenovirus vector for a TNF inhibitor construct.

In this study, Ad-mediated transfer of the vIL-10 gene resulted in transient expression and secretion of this anti-inflammatory cytokine into tears. These results are similar to a previous study in which we used the same model to evaluate the prophylactic action of an adenovirus vector construct with a TNF inhibitor gene (AdTNFRI).[33] In both studies, the transgene

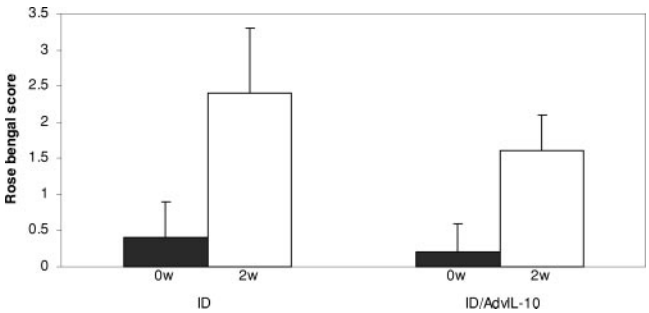

FIGURE 4. Rose bengal stain. Detection of deficiency in preocular tear film protection with rose bengal stain was determined in the ID group and compared with that in the ID/AdvIL-10–treated group. No difference was found between the two groups ($P = 0.35$).

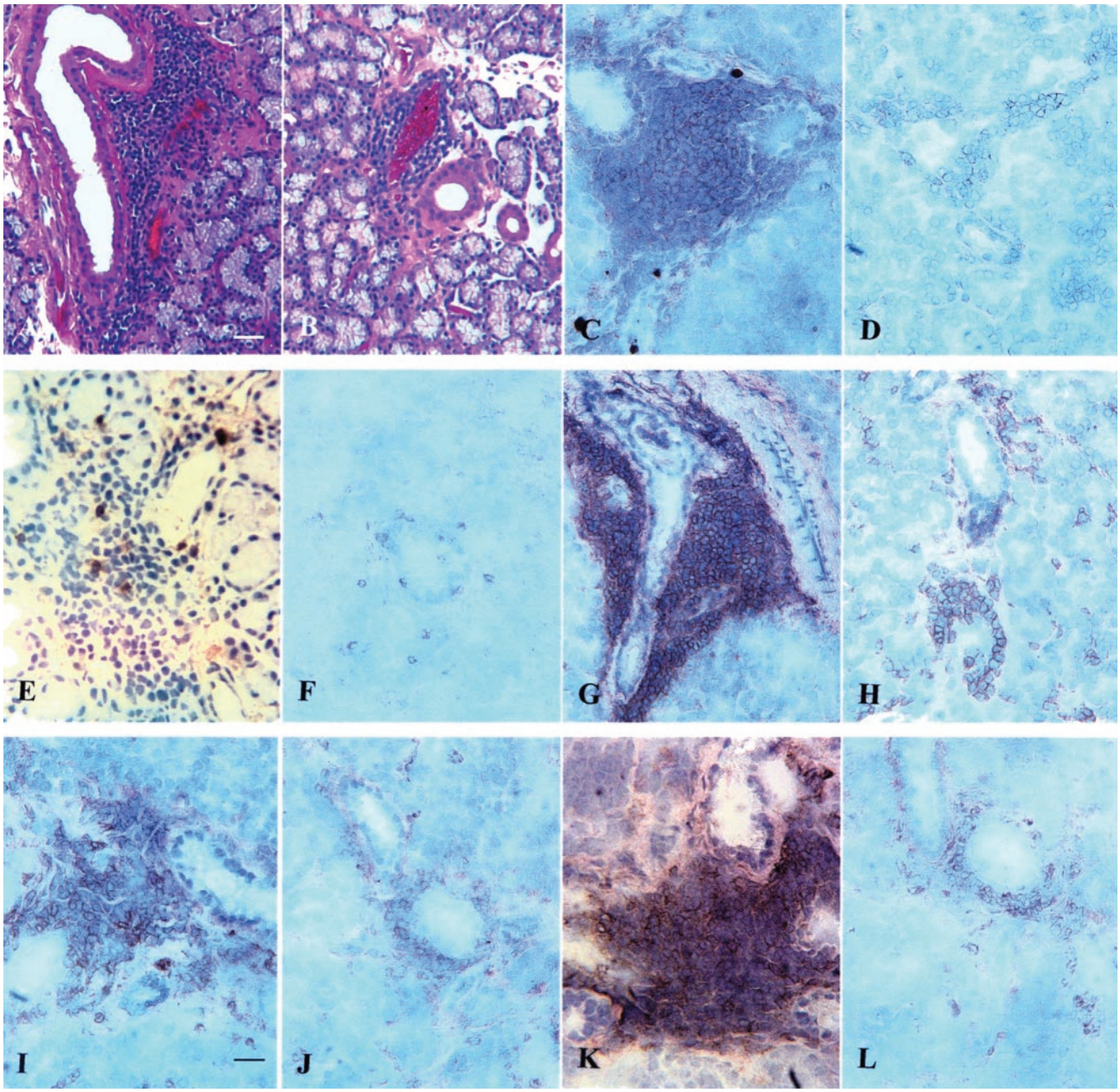

FIGURE 5. (**A**, **B**) Hematoxylin and eosin–stained lacrimal sections from animals designated ID and ID/AdvIL-10, respectively. (**C**, **D**) Immunohistochemical staining of similar sections for CD4, (**E**, **F**) CD8, (**G**, **H**) RTLA, (**I**, **J**) CD18, and (**K**, **L**) MHC-II. Bar: (**A**, **B**) 40 μm; (**C**–**L**) 20 μm.

products were secreted into the tears for less than 2 weeks. Such transient expression was expected because transferred genes are not integrated when adenovirus is used as the vector. Short-lived Ad-mediated vIL-10 expression has also been reported by De Kozak et al.[26]

Tear production and tear stability declined significantly in ID animals that did not receive AdvIL-10. These changes, along with a significant increase in rose bengal score, indicating ocular surface defects, represent a classic clinical picture of dry eye syndrome. In contrast, the clinical outcome after prophylactic treatment with AdvIL-10 was favorable in that tear production and tear stability were stabilized in animals treated with either AdIL-10 or AdTNFRI, whereas rose bengal scores, although not normal, were not as severely increased as in ID animals.

The model of induced autoimmune dacryoadenitis we have developed is characterized by significantly increased numbers of $CD4^+$, $CD18^+$, and $RTLA^+$ cells, but there was no increase in the number of $CD8^+$ cells. The number of MHC class II molecule+cells also increased in rabbits with induced autoimmune dacryoadenitis, in part because of the increased infiltration by activated lymphocytes and macrophages and in part because of induction of expression by ductal epithelial cells. Expression of the vIL-10 gene was associated with significantly decreased size and number of immune infiltrates, due to decreases in $CD4^+$, $CD18^+$, and RTLA cells. vIL-10 expression also was associated with a significant decrease in the number of MHC class II molecule-expressing cells, but with a significant increase in the number of $CD8^+$ cells.

TABLE 2. Immunohistochemical Analysis of Inflammatory Cells in Normal Lacrimal Glands, Glands with ID and ID/AdvIL-10–Treated Glands

Group	CD4	CD8	RTLA	CD18	MHCII
Normal	0.3 ± 0.1	0.5 ± 0.2	1.6 ± 0.8	0.2 ± 0.1	0.3 ± 0.1
ID	1.2 ± 0.5	0.3 ± 0.1	13.6 ± 5.7	12.1 ± 4.6	6.9 ± 3.3
ID/AdIL-10	0.6 ± 0.3	0.8 ± 0.4	8.7 ± 2.3	3.0 ± 1.4	2.6 ± 1.5
ANOVA *P*	0.0001	0.005	<0.0001	<0.0001	<0.0001
Pairwise comparisons*					
Normal vs. ID	<0.0001	0.450	<0.0001	<0.0001	<0.0001
ID vs. ID/AdvIL-10	0.0080	0.004	0.0200	<0.0001	0.0020
Normal vs. ID/AdvIL-10	0.1400	0.120	0.0004	0.0900	0.1700

Lacrimal glands were removed from each group 2 weeks after injection. For each antigen studied, five samples from each group were stained by the ABC method (four sections per sample) and analyzed by a cell image analysis system. Data are shown as mean positive percentage ± SD.

* Pairwise comparison probabilities, with a Bonferroni correction.

The role of IL-10 in SjS is still controversial. An early study failed to detect IL-10 mRNA in salivary glands of patients with primary SjS,[34] but subsequent investigations have yielded positive results.[35] It appears that serum IL-10 levels are elevated and correlate with increased autoantibody production and lymphocytic infiltration of the salivary glands in SjS.[36] IL-10 has been reported to be elevated in salivary glands of the NOD mouse model.[37] There have been conflicting reports concerning IL-10 expression in salivary glands of the MRL/Mp-lpr/lpr (MRL/lpr, MRL/MpJ-fas/fas) mouse,[38,39] and a report that it is elevated in lacrimal glands of both MRL/+ (MRL/MpJ-fas$^+$/fas$^+$) and MRL/lpr (MRL/MpJ-fas/fas) mice.[40] Using a mouse model transgenic for IL-10 under the amylase promoter, Saito et al.[41,42] found evidence that IL-10 induces apoptosis in glandular epithelial cells by increasing Fas ligand expression by infiltrating CD4$^+$ T cells. However, the immunopathology of this model differs from that in SjS and the autoimmune murine models. Moreover a role for Fas-ligand–mediated epithelial cell apoptosis in SjS is unlikely,[49] despite the documented increase in Fas expression.[44]

The effects we have reported are in agreement with published data showing that vIL-10 exerts its immunosuppressive properties by downregulating the MHC-II molecule and proinflammatory cytokine expression without stimulating cytotoxic T cells.[20,21,30] It is unclear how the beneficial anti-inflammatory effect of vIL-10 outlasts the detectable presence of the cytokine. One possible explanation is suggested by the observation that CD8$^+$ cells outnumbered CD4$^+$ cells in the normal human lacrimal gland by a ratio of 2:1, whereas the ratio decreased to 1:4 in both in SjS and in our model of induced autoimmune dacryoadenitis. In the ID/AdvIL-10–treated glands, the CD8$^+$-to-CD4$^+$ ratio decreased to only 1.3:1. We have suggested that activated CD8$^+$ cells in the normal gland may play a role as T regulatory cells in maintaining normal peripheral tolerance.[45] Therefore, the increased presence of vIL-10 after gene therapy in the gland may augment a favorable immunoregulatory response. Whereas the immunogenicity of adenovectors and the transience of adenovirus-mediated gene expression preclude their use for gene transfer therapy in humans, the results we have obtained suggest that vIL-10 may be beneficial if an appropriate delivery method can be devised.

CONCLUSIONS

In this study, adenovirus-mediated vIL-10 gene transduction resulted in the transient appearance of vIL-10 in tears. The presence of vIL-10 protein in tears suppressed both the clinical features of dry eye disease and lymphocytic infiltration associated with the experimental model of autoimmune dacryoadenitis.

Acknowledgments

The authors thank Michael Pidgeon for technical assistance and René de Waal Malefyt (DNAX, Palo Alto, CA), for kindly providing the vIL-10 cDNA.

References

1. Talal N. What is Sjögren's syndrome and why is it important? *J Rheumatol Suppl*. 2000;61:1–3.
2. Fox RI. Sjögren's syndrome. *Curr Opin Rheumatol*. 1995;7:409–416.
3. Sullivan DA, Wickham LA, Krenzer KL, Rocha EM, Toda I. Aqueous tear deficiency in Sjögren's syndrome: possible causes and potential treatment. In: Pleyer U, Hartmann C, Sterry W, eds. *Oculodermal Diseases*: *Immunology of Bullous Oculo-muco-cutaneous Disorders*. Buren, The Netherlands: Aeolus Press; 1997:95–152.
4. Esch TR. Pathogenetic factors in Sjögren's syndrome: recent developments. *Crit Rev Oral Biol Med*. 2001;12:244–251.
5. Kolkowski EC, Reth P, Pelusa F, et al. Th1 predominance and perforin expression in minor salivary glands from patients with primary Sjögren's syndrome. *J Autoimmun*. 1999;13:155–162.
6. Sun D, Emmert-Buck MR, Fox PC. Differential cytokine mRNA expression in human labial minor salivary glands in primary Sjögren's syndrome. *Autoimmunity*. 1998;28:125–137.
7. Magnusson V, Nakken B, Bolstad AI, Alarcon-Riquelme ME. Cytokine polymorphisms in systemic lupus erythematosus and Sjögren's syndrome. *Scand J Immunol*. 2001;54:55–61.
8. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry eye disease. *Invest Ophthalmol Vis Sci*. 2001;42:2283–2292.
9. Boumba D, Skopouli FN, Moutsopoulos HM. Cytokine mRNA expression in the labial salivary gland tissues from patients with primary Sjögren's syndrome. *Br J Rheumatol*. 1995;34:326–333.
10. Mitsias DI, Tzioufas AG, Veiopoulou C, et al. The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjögren's syndrome. *Clin Exp Immunol*. 2002;128:562–568.
11. De Vita S, Dolcetti R, Ferraccioli G, et al. Local cytokine expression in the progression toward B cell malignancy in Sjögren's syndrome. *J Rheumatol*. 1995;22:1674–1680.
12. Fox RI, Kang HI, Ando D, et al. Cytokine mRNA expression in salivary gland biopsies of Sjögren's syndrome. *J Immunol*. 1994;152:5532–5539.
13. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*. 1989;170:2081–2095.

14. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001; 19:683–765.
15. Akdis CA, Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology*. 2001;103:131–136.
16. Go NF, Castle BE, Barrett R, et al. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med*. 1990;172:1625–1631.
17. Vieira P, de Waal-Malefyt R, Dang MN, et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc Natl Acad Sci USA*. 1991;88:1172–1176.
18. Jorgensen C, Apparailly F, Sany J. Immunological evaluation of cytokine and anticytokine immunotherapy in vivo: what have we learnt? *Ann Rheum Dis*. 1999;58:136–141.
19. Asadullah K, Sterry W, Stephanek K, et al. IL10 is a key cytokine in psoriasis: proof of principle by IL-10 therapy—a new therapeutic approach. *J Clin Invest*. 1998;101:783–794.
20. Zhu Z, Stevenson D, Ritter T, et al. Expression of IL-10 and TNF-inhibitor genes in lacrimal gland epithelial cells suppresses their ability to activate lymphocytes. *Cornea*. 2002;21:210–214.
21. Zhu Z, Stevenson D, Schechter JE, Mircheff AK, Atkinson R, Trousdale MD. Lacrimal histopathology and ocular surface disease in a rabbit model of autoimmune dacryoadenitis. *Cornea*. 2003;22:25–32.
22. Pretolani M. Interleukin-10: an anti-inflammatory cytokine with therapeutic potential. *Clin Exp Allergy*. 1999;29:1164–1171.
23. Mottonen M, Isomaki P, Saario R, Toivanen P, Punnonen J, Lassila O. Interleukin-10 inhibits the capacity of synovial macrophages to function as antigen-presenting cells. *Br J Rheumatol*. 1998;37: 1207–1214.
24. Ferguson TA. The molecular basis of anterior associated immune deviation (ACAID). *Ocul Immunol Inflamm*. 1997;5:213–215.
25. Balasa B, La Cava A, Van Gunst K, et al. A mechanism for IL-10-mediated diabetes in the nonobese diabetic (NOD) mouse: ICAM-1 deficiency blocks accelerated diabetes. *J Immunol*. 2000;165: 7330–7337.
26. De Kozak Y, Thillaye-Goldenberg B, Naud MC, Da Costa AV, Auriault C, Verwaerde C. Inhibition of experimental autoimmune uveoretinitis by systemic and subconjunctival adenovirus-mediated transfer of the viral IL-10 gene. *Clin Exp Immunol*. 2002;130: 212–223.
27. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. *Arthritis Rheum*. 2001; 44:3–12.
28. Rizzo LV, Xu H, Chan CC, Wiggert B, Caspi RR. IL-10 has a protective role in experimental autoimmune uveoretinitis. *Int Immunol*. 1998;10:807–814.
29. Apparailly F, Verwaerde C, Jacquet C, Auriault C, Sany J, Jorgensen C. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. *J Immunol*. 1998;160:5213–5220.
30. Ma Y, Thornton S, Duwel LE, et al. Inhibition of collagen-induced arthritis in mice by viral Il-10 gene transfer. *J Immunol*. 1998;161: 1516–1524.
31. Kaya Z, Dohmen KM, Wang Y, et al. Cutting edge: a critical role for IL-10 in induction of nasal tolerance in experimental autoimmune myocarditis. *J Immunol*. 2002;168:1552–1556.
32. Klebe S, Sykes P, Coster D, Krishnan R, Williams K. Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. *Transplantation*. 2001;71:1214–1220.
33. Zhu Z, Stevenson D, Schechter JE, et al. Tumor necrosis factor inhibitor gene expression suppresses lacrimal gland immunopathology in a rabbit model of autoimmune dacryoadenitis. *Cornea*. 2003;22:343–351.
34. Boumba D, Skopouli FN, Moutsopoulos HM. Cytokine mRNA expression in the labial salivary gland tissues from patients with primary Sjögren's syndrome. *Br J Rheumatol.* 1995;34:326–333.
35. Garcic-Carrasco M, Font J, Filella X, et al. Circulating levels of Th1/Th2 cytokines in patients with primary Sjögren's syndrome: correlation with clinical and immunological features. *Clin Exp Rheumatol*. 2001;19:411–415.
36. Perrier S, Serre AF, Dubost JJ, et al. Increased serum levels of interleukin 10 in Sjögren's syndrome; correlation with increased IgG1. *J Rheumatol*. 2000;27:935–939.
37. Yanagi K, Ishimaru N, Haneji N, et al. Anti-120-kDa alpha fodrin immune response with Th1 cytokine profile in the NOD mouse model of Sjögren's syndrome. *Eur J Immunol*. 1998;28:3336–3345.
38. Mustafa W, Zhu J, Deng G, et al. Augmented levels of macrophage and Th1 cell-related cytokine mRNA in submandibular glands of MRL/lpr mice with autoimmune sialadenitis. *Clin Exp Immunol*. 1998;112:389–396.
39. Yanagi K, Haneji N, Hamano H, Takahashi M, Higashima H, Hayashi Y. In vivo role of IL-10 and IL-12 during development of Sjögren's syndrome in MRL/lpr mice. *Cell Immunol*. 1996;168: 243–250.
40. Jabs DA, Prendergast RA, Rorer EM. Hudson AP, Whittum-Hudson JA. Cytokines in autoimmune lacrimal gland disease in MRL/mpJ mice. *Invest Ophthalmol Vis Sci.* 2001;42:2567–2571.
41. Saito I. Structure of IL-10 and its role in autoimmune exocrinopathy. *Crit Rev Immunol*. 2000;20:153–165.
42. Saito I, Haruta K, Shimuta M, et al. Fas ligand-mediated exocrinopathy resembling Sjögren's syndrome in mice transgenic for IL-10. *J Immunol*. 1999;162:2488–2494.
43. Ohlsson M, Skarstein K, Blostad AI, et al. Fas-induced apoptosis is a rare event in Sjögren's syndrome. *Lab Invest*. 2001;81:95–105.
44. Bolstad AI, Eiken JG, Rosenlund B, et al. Increased salivary gland tissue expression of Fas, Fas ligand, cytotoxic lymphocyte-associated antigen-4, and programmed cell death 1 in primary Sjögren's syndrome. *Arthritis Rheum.* 2003;48:174–185.
45. Mircheff AK, Hamm-Alvarez SF, Kaslow HR, Schechter JE, Trousdale MD. Epithelial cells in lymphocyte regulation in the lacrimal gland. In: Zierhut M, ed. *Immunology of the Ocular Surface and the Lacrimal Glands.* Amsterdam: Swets & Zeitlinger. In press.