Pharmacological Cholinergic Blockade Stimulates Inflammatory Cytokine Production and Lymphocytic Infiltration in the Mouse Lacrimal Gland

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PURPOSE. To investigate the effects of cholinergic blockade on inflammatory cell infiltration and cytokine production in the mouse lacrimal gland (LG).

METHODS. C57BL/6 mice were untreated (UT) or received subcutaneous injections of either scopolamine hydrobromide (SCOP; 0.5 mg/0.2 mL) or saline (SAL) four times daily for 2 or 5 days (2D, 5D). This was followed by a 7-day rest period in separate groups. Tear volume (cotton thread) and tear epidermal growth factor (EGF, by ELISA) concentrations were measured. Extraorbital LGs were surgically excised and sectioned or lysed for gene expression analysis. Immunohistochemistry evaluated immunophenotype of infiltrating cells. Expression of EGF and T helper (Th)-1, -2, and -17-associated cytokines in LGs was evaluated by real-time PCR. Goblet cell density was evaluated in periodic acid Schiff-stained conjunctival sections.

RESULTS. Tear volume and EGF protein levels were significantly reduced in SCOP5D mice compared with controls, indicating that cholinergic blockade decreased LG secretory function. LGs of SCOP2D and SCOP5D mice showed an increased density of CD4⁺, CD11c⁺, CD11b⁺, and myeloperoxidase⁺ cells compared with UT controls. At day 5, these cells were significantly elevated compared with SAL-treated counterparts. Elevated levels of IL-17A, IL-17R, IFN- γ , IL-12R β 1, IL-2, IL-13, IL-6, IL-1 β , and TNF- α transcripts were noted in SCOP2D mice and IFN- γ , TGF- β 1, and IL-18R transcripts in SCOP5D mice.

CONCLUSIONS. Pharmacological blockade of lacrimal secretion induced a significant CD4⁺ infiltration in the LG, mimicking Sjögren's syndrome. The mRNA expression profile revealed elevations of a mix of inflammatory cytokines and Th-1-associated factors. (*Invest Ophthalmol Vis Sci.* 2011;52:3221-3227) DOI:10.1167/iovs.09-4212

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The lacrimal gland (LG) is a dynamic organ that secretes proteins, electrolytes, and water to protect and support the ocular surface.¹ The functional importance of the LG is reflected by its highly developed neural regulation. Stimulus of ocular surface trigeminal afferents activates a reflex arc that ultimately leads to LG secretion via efferent parasympathetic and sympathetic nerves.² Acetylcholine, a potent cholinergic neurotransmitter stimulus of LG secretion, operates by initially binding to the M3 subtype muscarinic receptor (M3AChR).³

Dysfunction of the LG is the primary cause of aqueous tear deficiency, a common subtype of dry eye disease (DED). Sjögren's syndrome (SS), an autoimmune disease that affects an estimated 1.3 million Americans,⁴ causes the most severe aqueous tear deficient DED.⁵ The disease affects women nine times more frequently than men, and the onset is commonly in the fourth to sixth decades. Patients with SS present with an array of symptoms related to diminished LG and salivary gland function, including keratoconjunctivitis sicca, xerostomia, and enlargement of the parotid gland. SS can occur alone (primary SS) or in the presence of other autoimmune diseases (secondary SS), such as systemic lupus erythematosus, rheumatoid arthritis, or scleroderma. Truly a systemic disease, SS is associated with clinically significant musculoskeletal, dermatologic, pulmonary, renal, gastrointestinal, and hematologic involvement.⁶

Despite well-defined clinical characteristics, the autoimmune basis of SS is complex and remains unclear. Many studies have demonstrated focal, periductal, and perivascular lymphocytic infiltrates in the LGs of SS patients.^{7,8} Nearly complete loss of secretory function may be observed in many patients early in the course of the disease when there is mild to moderate parenchymal destruction. This discrepancy has increased interest in the pathophysiological significance of autoantibodies to the M3AChR, which inhibit parasympathetic neurotransmission and are present in the sera of patients with SS.^{9,10} In rabbit LGs, M3AChR antagonists have been shown to suppress protein secretion.¹¹ Transgenic mice lacking the M3AChR display reduced secretory response to cholinergic stimulation.¹² Additionally, incubation of LG lobules with IL-1 β , a cytokine that is upregulated in a murine model of SS, was found to inhibit neurally mediated peroxidase secretion.¹³ These actions at the cell-signaling level may explain loss of LG function in the presence of relatively preserved architecture.

An experimental approach that has been used to modulate parasympathetic signaling in the LG is the surgical removal of the preganglionic nerve responsible for the efferent arm of the lacrimation reflex.¹⁴⁻¹⁶ This technique anatomically eliminates the acetylcholine needed for stimulation of muscarinic receptors on the membranes of LG acinar cells. Experimental rat and rabbit models using this technique have demonstrated dramatic reduction in tear production, profound changes to LG

structure, and significant pathologic changes on the ocular surface. Cholinergic denervation has been accompanied by massive accumulation of secretory granules in the LG acinar cells,¹⁴ visible by electron microscopy, and upregulated expression of pro-inflammatory genes.^{15,16}

Another method of parasympathetic inhibition is by pharmacologic blockade. In rats, systemic scopolamine injection has been shown to produce significant secondary alterations in the corneal epithelium, decreased MUC5AC immunoreactivity in the conjunctival epithelium, and modification of extraorbital LG fatty acid profile and cytokine (IL-1 β , IL-6, TNF- α) expression.¹⁷ In the present study, we use a previously reported method of cholinergic pharmacologic blockade in C57BL/6 mice and characterize the subsequent changes in LG function, histopathology, and local immune environment.

METHODS

Mice

C57BL/6 mice, 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). All animal experiments were approved by the institutional animal care and use committees at Baylor College of Medicine. All studies adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research.

Pharmacologic Cholinergic Blockade

Cholinergic inhibition was induced by subcutaneous injection of scopolamine hydrobromide (SCOP, 0.5 mg/0.2 mL; Sigma-Aldrich, St. Louis, MO) three times a day (0830, 1330, and 1700 hours) alternating between the right and left flanks of 8-week-old mice for a total of 2 (SCOP2D) or 5 (SCOP5D) days. Control mice were similarly injected with saline (SAL) at the same volume, frequency, and duration (SAL2D and SAL5D, respectively). Subsets of SCOP5D and SAL5D mice were also studied after 7 days free from injections after the initial treatment. These experimental groups were compared with mice receiving no treatment at all (untreated [UT]).

Histology and Periodic Acid Schiff Staining and Goblet Cell Measurement

Enucleated mouse eyes, submandibular glands, and small intestine from all treatment groups ($n = 6$) were surgically excised and fixed in 10% formalin and embedded in paraffin. Eight-micrometer sections were cut and stained with hematoxylin and eosin (H&E) for evaluation of morphology. Goblet cell density was evaluated in sections stained with periodic acid Schiff (PAS). Sections from 6 different left eyes in each group were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon, Melville, NY). Goblet cell density in the superior and inferior conjunctiva was measured in six eyes of each group using imaging software (Metavue 6.24r software; Molecular Devices, Sunnyvale, CA).

Immunohistochemistry

For immunohistochemistry, extraorbital LGs of each group and time point ($n = 5$) were excised, embedded in optimal cutting temperature compound (VWR; Suwanee, GA), and flash frozen in liquid nitrogen. Sagittal 8- μ m sections were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and placed on glass slides that were stored at -80°C .

Immunohistochemistry was performed to detect and count the cells in the LG that stained positively for CD4 (clone H129.9, 10 $\mu\text{g}/\text{mL}$; BD Bioscience, San Diego, CA), CD8 α (clone 53e6.7, 3.125 $\mu\text{g}/\text{mL}$; BD Bioscience), CD11b (clone M1/70, 6.25 $\mu\text{g}/\text{mL}$; BD Bioscience), CD45 (clone 30-F11, 10 $\mu\text{g}/\text{mL}$; BD Bioscience), CD11c (clone HL3, 12.5 $\mu\text{g}/\text{mL}$; BD Bioscience), CD103 (clone 2E7, 10 $\mu\text{g}/\text{mL}$; Biolegend, San Diego, CA), and myeloperoxidase (1:300 of neat serum;

Neomarkers, Fremont, CA). Cryosections were stained with the above mentioned primary antibodies and appropriate biotinylated secondary antibodies (all from BD Pharmingen, San Diego, CA) using a staining kit (VectastainElite ABC kit; Vector, Burlingame, CA) and reagents (Nova-Red; Vector). Secondary antibody alone and appropriate anti-mouse isotype (BD Biosciences) controls were also performed. Two sections from each animal were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon). Positively stained cells were counted in the stroma of the LG using image-analysis software (NIS Elements Software, version 3.0 BR; Nikon). Results were expressed as the number of positive cells per area (mm^2).

Tear Volume Measurement and EGF ELISA

Aqueous tear production was assessed using a cotton thread (Quick Thread; FCI Ophthalmics, Marshfield Hills, MA) as previously described.^{18,19} Tear fluid washings were collected from 20 animals/group, in three independent experiments, using a previously reported method.¹⁸ One sample consisted of tear washings from both eyes of one mouse that were pooled (2 μL total) in PBS+0.1% BSA (8 μL) and stored at -80°C until the assay was performed.

Mouse EGF was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. There were a total of six samples per group. Results are presented as percentage change from baseline.

Isolation of Cervical Lymph Nodes

Superficial and deep cervical lymph nodes (CLNs) from each group were surgically excised, smashed between two sterile frosted glass slides, and made into a single-cell suspension. Cell populations were individually collected, centrifuged at 1000 rpm for 5 minutes, filtered, and resuspended. Single cells were either processed for flow cytometry or were lysed for RNA analysis, as described below.

Flow Cytometry Analysis of Murine Cells

Single-cell suspensions from superficial and deep CLN were stained with anti-CD16/32 (to block Fc receptors; BD Pharmingen), followed by cell surface staining with FITC-anti-CD4 (GK1.5; BD Pharmingen). Negative controls consisted of cells stained with FITC-isotype antibody (BD Pharmingen). Cells were then resuspended in fixation-permeabilization solution (Cytofix/Cytoperm; BD Pharmingen). A BD LSRII benchtop cytometer was used for flow cytometry, and data were analyzed using BD Diva Software (BD Pharmingen). These experiments were repeated twice.

RNA Isolation and Real-Time PCR

Total RNA from the LGs (individually collected and individually pooled) or single-cell suspension from CLN of all treatment groups was extracted using an acid guanidiumthiocyanate-phenol-chloroform method as previously described.²⁰ Four samples per treatment group were used, and one tube consisted of pooled extracts from 5 mice. Samples were treated with DNase to prevent genomic DNA contamination according to the manufacturer's instructions (Qiagen, Valencia, CA).

First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers and M-MuLV reverse transcriptase (Ready-to-Go You-Prime First-Strand Beads; GE Health Care, Piscataway, NJ), as previously described.²¹ Real-time PCR was performed using gene expression assay primers and MGB probes specific for murine GAPDH, MMP-9, IL-6, TGF- β 1, TGF- β 2, IL-17A, IL-23 receptor, IL-17 receptor, ROR γ T, IFN- γ , IL-2, IL-12, T-bet, IL-12 receptor β 1, IL-18 receptor, IL-4, IL-13, and EGF (Assay IDs: Mm99999915, Mm01240564, Mm00446490, Mm004417241, Mm00436952, Mm00439619, Mm00519942, Mm00434214, Mm00441139, Mm00801778, Mm00434256, Mm00434165, Mm00450960, Mm01351787, Mm01233989, Mm00445259, Mm00434204, and Mm01187875, respectively). The

FIGURE 3. Mouse submandibular gland (A, SMG) and small intestine (B) sections from untreated (UT), saline-treated 2 days (2D SAL), 5 days (5D SAL), and 5 days then rest (5D SAL + rest), and scopolamine-treated 2 days (2D SCOP), 5 days (5D SCOP), and 7 days of rest (5D SCOP + rest) stained with H&E.

we also evaluated goblet cell density in PAS-stained conjunctival sections. There was no change from baseline at all time points in the scopolamine-treated group, and the GC densities at all time points were comparable to the saline-injected control group (data not shown).

Because systemic administration of scopolamine could also affect innervation of lymph nodes resulting in a nonspecific inflammatory response, we performed flow cytometry analysis of cervical lymph node cells stained for CD4. As shown in

Figure 2A, similar percentages of CD4⁺ lymphocytes were observed in the cervical lymph nodes in all groups.

To address the concern that nonspecific inflammation due to cholinergic blockade could be present in other organs, we also evaluated submandibular gland and small intestine histology. There was no difference in the histologic appearance of these organs after treatment (Fig. 3). Furthermore, there was no change in CD4⁺T cell density after treatment (data not shown).

FIGURE 4. Mouse lacrimal gland sections from untreated (UT), saline-treated 2 days (2D SAL), 5 days (5D SAL), and 5 days then rest (5D SAL + rest), and scopolamine-treated 2 days (2D SCOP), 5 days (5D SCOP), and 7 days of rest (5D SCOP + rest) stained with H&E (A) or immunostained for CD4 (B). The areas circumscribed in (A) are shown in high magnification to show T cell infiltration (arrows). NC, negative control of UT LG stained for CD4.

Taken together, these results indicate that systemic cholinergic blockade does not induce a nonspecific inflammation in four different sites that receive cholinergic innervation (conjunctival goblet cells, cervical lymph nodes, submandibular glands, and small intestine).

Cholinergic Blockade Induces Inflammatory Infiltration of the Lacrimal Gland

After identifying that cholinergic blockade was present and not inducing nonspecific systemic immune responses, we evaluated inflammatory changes in the LG by immunohistochemistry and H&E staining (Figs. 4, 5).

C57BL/6 mice injected with SCOP displayed inflammatory cell infiltration in the parenchyma of the LG, as early as 2 days after treatment, compared to SAL and UT groups (Fig. 4). Many of these cells were noted in a periductal distribution, and they stained positively for CD4. Individual cell types were identified

and counted after immunohistochemical staining (see individual counts in Fig. 5). After 2 days of SCOP, numbers of CD11b (a marker of monocyte/macrophage lineage) and myeloperoxidase (a marker of neutrophils) positive (+) cells were significantly increased, and CD8 α + cells were decreased compared to SAL treatment. At 5 days of SCOP treatment, there were significantly more cells stained for CD4, CD11c (dendritic cell marker), CD103 (α E integrin, a marker of intraepithelial lymphocytes), and CD11b than SAL treatment. After 7 days of rest after the last SCOP injection, the number of most of the infiltrating cells decreased in relation to the 5-day time point; however, they still remained elevated compared to UT and SAL-treated groups. The only exception was CD103+ cells, whose numbers continued to increase from 5 days. These data combined with our other findings suggest the increase in CD45+ cells in the saline group post rest is not due to neutrophils or T cells. We also noted an increase in CD11b+ monocytic cells in the saline-treated groups after the rest, which could account for the higher number of CD45+ cells.

Collectively, these results indicate that cholinergic blockade promotes infiltration of the LG with inflammatory cells as early as 2 days, with a peak at 5 days, and some persistent inflammation even after release of cholinergic blockade.

Cholinergic Blockade Stimulates Inflammatory Cytokine Production in the Lacrimal Gland but Not in Cervical Lymph Nodes

Expression levels of T cell-associated transcripts and factors were evaluated to further characterize the phenotype of the inflammatory cells infiltrating the LG. These results are presented in Figure 6. Levels of the Th-17-associated factors IL-6, TGF β -1, and IL-17A were significantly elevated in mice treated with SCOP2D compared with both UT and SAL2D groups (Fig. 6A). This was associated with a concomitant peak of the inflammatory cytokines IL-1 β and TNF- α transcripts (Fig. 6B). TGF β -1 mRNA levels peaked after 5 days of scopolamine treatments but remained elevated after 7 days of rest. Levels of Th-1 (IFN- γ , IL-2, IL-12R β 1, and IL-18R, T-bet) mRNA transcripts were also significantly elevated in the SCOP2D-treated group compared with both control groups (Fig. 6C). IFN- γ , IL-18R, and T-bet remained elevated at 5 days of scopolamine treatment. MMP-9, TGF- β 2, ROR γ T, and IL-4 did not change significantly at any measured time point.

We also evaluated the T cell-associated profile in cells from CLN of both treatment groups at days 2, 5, and rest time points, using real-time PCR. To evaluate specificity, we investigated some of the same genes that were found to be elevated in the LG. Our results demonstrate no difference in levels of IL-17A, IFN- γ , TGF- β 1, IL-1 β , and TNF- α mRNA transcripts between baseline and treatment groups (Fig. 2B).

These results indicate that cholinergic blockade induces an inflammatory response with accumulation of T cells and release of T cell-related cytokines in the lacrimal gland, but not in the regional lymph nodes.

DISCUSSION

This study was performed to investigate if inhibition of secretory function by systemic cholinergic blockade had pro-inflammatory effects in the lacrimal gland. It is now recognized that patients with the severe autoimmune dry eye condition SS have significantly higher levels of circulating autoantibodies to the M3 acetylcholine receptor than normal subjects and these autoantibodies have been shown to inhibit cholinergic stimulated glandular secretion.^{24,25} We found that pharmacological cholinergic inhibition resulted in LG inflammation in the mouse. We observed an influx of CD4 $^{+}$ T cells into the paren-

FIGURE 5. Cell counts in mouse lacrimal glands stained by immunohistochemistry for CD4, CD8, CD11c, CD103, CD11b, CD45, and myeloperoxidase sections in untreated (UT), saline-treated, and scopolamine-treated mice at 2 days (2D), 5 days (5D), and 7 days of rest (rest). * $P < 0.001$, $^{\wedge}P < 0.01$, $^{\sim}P < 0.05$.

FIGURE 6. mRNA transcript levels of Th-17 pathway (A), IL-1 β and TNF- α inflammatory cytokines (B), and Th-1 and Th-2 pathways (C), in lacrimal glands of untreated (UT), saline-treated 2 days (SAL 2D), 5 days (SAL5D), and 7 days of rest (SAL5D + rest), and scopolamine-treated 2 days (SCOP2D), 5 days (SCOP5D), and 7 days of rest (SCOP5D + rest). * P < 0.05, % P < 0.01, $\$P$ < 0.001, $\#P$ < 0.0001.

chyma and periductal regions of the LG, similar to the pattern seen in SS.^{7,8} Dendritic cells, naïve T cells, neutrophils, and cells of monocyte lineage were also increased in mice treated with SCOP. There was still an increase of bone marrow-derived cells at the rest time point in the saline-treated group, which was not due to an increase of neutrophils. We also noted an increase in CD11b⁺ monocytic cells in the saline-treated groups after the rest, which could account for some of the higher number of CD45⁺ cells. These differences were significant compared with SAL-treated and UT controls, especially after 5 days of scopolamine treatment. The identity of the remaining cells accountable for the increase in the CD45⁺ cells remains to be determined. Neither necrosis nor significant alteration of lacrimal gland structure was observed.

Tear volume and EGF concentration, markers of LG secretory activity, were noted to decrease after 5 days of pharmacologic cholinergic inhibition in C57BL/6 mice. This is consistent with studies of LG denervation in rabbits, which demonstrated a 60% reduction in tear flow within 24 hours.¹⁴ Ultrastructural examination of LG acinar cells in these models showed numerous irregular secretory granules, suggesting limited exocytotic capacity and impaired intracellular vesicle and cell membrane trafficking. It is plausible to suggest that pharmacologic cholinergic blockade produces similar effects in the acinar cells. Secretory blockage could lead to greater exposure of lacrimal autoantigens to resident antigen presenting cells and initiate an autoimmune reaction. Indeed, Mircheff and colleagues have proposed that LG epitopes recognized by autoreactive lymphocytes are constitutively available and that low-grade autoimmunity is the normal state in the lacrimal gland, kept in check by immunoregulatory mechanisms.²⁶ Our

findings suggest that perturbation of normal secretory processes may upset this balance and stimulate lacrimal inflammation. This mechanism could explain the development of lacrimal dysfunction in a variety of conditions (e.g., trigeminal nerve ablation, herpes zoster ophthalmicus, and post-LASIK) where neural signaling pathways involved in tear secretion are disrupted.^{27–29} It remains to be determined if the lacrimal dysfunction associated with these conditions has an autoimmune basis.

Our findings suggest that a possible mechanism by which cholinergic blockade induced by M3R autoantibodies in SS could contribute to lacrimal gland inflammation. Human SS sera from SS patients containing anti-M3R autoantibodies was found to functionally inhibit stimulated saliva secretion when administered to NOD.Igmu null mice.²⁵ Because these autoantibodies appear capable of inhibiting cholinergic signaling similar to anticholinergic agents such as scopolamine, it is possible that prolonged autoantibody-mediated cholinergic blockade could also promote lacrimal gland inflammation and secretory dysfunction. Obviously, this theory remains to be proved.

Consistent with the observed increase in CD4⁺ T cells was an increase in levels of T cell-associated factors in the LG after cholinergic blockade. Our examination of the profile of these genes in the LG after cholinergic inhibition revealed an interesting pattern of expression. At 2 days of treatment, significantly higher levels of IL-6 and TGF- β 1, accompanied by an increase in IL-17, is consistent with an early Th-17 response. We hypothesize that this early increase of IL-17A mRNA could be due to stimulation of innate resident immune cells, such as NK and $\gamma\delta$ T cells, because they have also been shown to produce IL-17.³⁰

Simultaneously, we observed significantly elevated expression of the inflammatory cytokines IL-1 β and TNF- α . Levels of the IFN- γ , IL-18R, and T-bet demonstrated an apparent peak at 5 days of scopolamine treatment, concomitant with the peak of infiltrating inflammatory cells. These findings are consistent with increases in T helper cytokines that have been observed in the lacrimal glands of C57BL/6.NOD-Aec1Aec2 mice that develop SS like disease.^{31,32} Elevated levels of IFN- γ , IL-4, and IL-17A have been found in this autoimmune prone strain.^{31,32} Elimination of IFN- γ in this model prevented glandular apoptosis and improved EGF production by the LG,³¹ while elimination of IL-4 preserved secretory function despite no apparent change in the density of glandular infiltration.³² The pathogenic role of IL-17 in this C57BL/6.NOD-Aec1Aec2 strain has yet to be determined. In contrast, LG infiltrates in the MRL/Mp mouse model of SS demonstrated elevated IL-4 immunostaining and mRNA transcript levels, suggesting a Th-2 response.³³⁻³⁵

Taken together, our findings suggest that pharmacological blockade of secretory function was sufficient to elicit a massive inflammatory infiltrate of the LG and increased production of inflammatory cytokines that, if sustained, could lead to loss of glandular tissue.

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