Inflammatory Mediators in Autoimmune Lacrimal Gland Disease in MRL/MpJ Mice

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PURPOSE. MRL/MpJ- fas+/fas+ (MRL/+ ) and MRL/MpJ- faslpr/faslpr (MRL/lpr) mice are congenic substrains of mice that have spontaneously developing lacrimal and salivary gland inflammation and are models for the human disorder Sjögren’s syndrome. Nitric oxide (NO) and tumor necrosis factor (TNF)-α are proinflammatory and potential mediators of tissue damage. The presence of the inducible form of nitric oxide synthase (iNOS), which catalyzes the production of NO, and the presence of TNF-α in the lacrimal glands of MRL/MpJ mice were assessed.

METHODS. Lacrimal glands from MRL/+ and MRL/lpr mice, at ages 1 through 9 months, were evaluated by real-time RT-PCR for iNOS and TNF-α mRNA and by immunohistochemistry for the presence of iNOS and of TNF-α. Age-matched BALB/c lacrimal glands were used as the control.

RESULTS. By quantitative real-time PCR (qPCR), mRNA for iNOS was detected in the lacrimal glands in significantly greater amounts in both MRL/+ (median, normalized to 18S rRNA, 2.90; P < 0.0003) and MRL/lpr mice (median 6.84, P < 0.001) than in BALB/c mice (median 0.54). By qPCR, mRNA for TNF-α in the lacrimal glands was detected in significantly greater amounts in aged MRL/+ mice than in BALB/c mice (median, normalized to actin, 221.8 vs. 77.8, P = 0.011) and in MRL/lpr mice than in BALB/c mice (median 136.7 vs. 72.5, P = 0.001). Immunohistochemistry demonstrated both iNOS and TNF-α in scattered mononuclear cells throughout the lacrimal glands and in mononuclear cells at the junction of the focal inflammatory infiltrates and normal acinar tissue in both MRL/+ and MRL/lpr mice.

CONCLUSIONS. As demonstrated by the greater presence of iNOS and TNF-α in the lacrimal glands of MRL/MpJ mice than in control glands, both NO and TNF-α are potential mediators of lacrimal gland damage in these murine models of Sjögren’s syndrome. (Invest Ophthalmol Vis Sci. 2004;45:2293–2298) DOI:10.1167/iovs.03-0958

Sjögren’s syndrome is a chronic autoimmune disorder characterized by keratoconjunctivitis sicca and xerostomia. There is a progressive loss of exocrine gland function due to glandular damage, which results from a mononuclear inflammatory cell infiltration of these target organs. The frequent coexistence of Sjögren’s syndrome with systemic connective tissue diseases, such as rheumatoid arthritis and systemic lupus erythematosus, suggests that it is an autoimmune disease.1 MRL/MpJ mice have spontaneously developing lacrimal and salivary gland inflammation and are a model of human Sjögren’s syndrome.2–4 MRL/MpJ- fas+/fas+ (MRL/+ ) and MRL/MpJ- faslpr/faslpr (MRL/lpr) mice are congenic, spontaneously autoimmune mice that differ only by a single autosomal recessive mutation.2,5 The faslpr mutation results in defective Fas protein, defective lymphocyte apoptosis in peripheral lymphoid organs, systemic autoimmune disease, and accelerated lacrimal gland inflammation in MRL/lpr mice.5

The lacrimal gland disease in these mice appears to be T-cell-mediated with a predominance of CD4+ T cells at the inflammatory site,3,6 and the disease can be transferred into SCID mice by CD4+ T cells isolated from the glandular tissue.6 We have reported that the lacrimal glands lesions in both substrains appear to be Th2-mediated. There is a substantially greater expression of the cytokines IL-4 and -10 than of IFN-γ and IL-12 by immunohistochemistry and by RT-PCR for mRNA, and there is a greater expression of the costimulatory molecule B7-2 (CD80) than of B7-1 (CD86), which are associated with Th2 and Th1 responses, respectively.7–9

Nitric oxide (NO) is a multifunctional molecule produced by several cell types. NO has several physiologic functions, such as relaxation of muscle cells, neurotransmission, and modulating hematopoietic cells. It also has potent proinflammatory effects and may cause tissue damage through a superoxide-hydroxyl radical pathway. The production of NO is catalyzed by nitric oxide synthase (NOS), and its production in inflammatory conditions is catalyzed by an inducible form of NOS (iNOS), also known as NOS2. NO is a short-lived molecule, and iNOS typically is assessed when evaluating the role of NO in inflammatory conditions.10,11

Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that mediates tissue damage in several immune-mediated disorders, including rheumatoid arthritis.12–15 Inhibition of TNF-α has been reported to be effective as therapy in patients with rheumatoid arthritis and Crohn’s disease,14–16 and there are suggestions that it may be an effective therapy in several types of uveitis.17–18

In this study, we evaluated the presence of iNOS and TNF-α in the lacrimal glands of MRL/MpJ mice to determine their potential role as mediators of inflammation and tissue damage in this murine model of Sjögren’s syndrome.
MATERIALS AND METHODS

Animals

MRL/MpJ mice of MRL/+) and MRL/lpr substrains and control BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME) at age 1 month and kept under standard conditions. Groups of 8 to 12 mice of each strain were anesthetized and killed by exsanguination at ages 1 to 1.5, 3, and 5 months. A group of MRL/+) and another of control BALB/c mice also were killed at age 9 months, but not of MRL/lpr mice, as they typically do not survive beyond 6 months. At the time of death, lacrimal glands were removed and processed either for quantitative real-time RT-PCR (qPCR) or for immunohistochemistry for iNOS or TNF-α. Lacrimal glands from three to eight mice of each age and strain were processed for real-time PCR and from five mice for immunohistochemistry. A portion of one lacrimal gland from each animal was evaluated by histopathology as well. These experiments were approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Evaluation of Lacrimal Gland Histology

Lacrimal gland sections were graded using a modified focus score scale, as previously described. With this scoring system, lacrimal gland sections were graded from 0 to 4, based on the presence of inflammatory foci consisting of 50 or more mononuclear inflammatory cells: grade 0, no inflammatory cells; grade 1, inflammatory cell infiltration without any foci; grade 2, the presence of at least one focus; grade 3, multiple foci; and grade 4 multiple foci plus evidence of lacrimal gland destruction.

Quantitative Real-Time RT-PCR

Total nucleic acids were isolated from tissues by homogenization with extraction reagent (TRIzol; Invitrogen-Gibco, San Diego, CA). Pure RNA was prepared by treatment of total nucleic acid preparations with DNase 1 (RQ1; Promega Life Sciences, Madison, WI), followed by phenol-chloroform extraction and ethanol precipitation. RNA preparations were assessed for residual DNA by standard PCR using primers designed to amplify the predicted products by qPCR as described by us before.

Statistics

The comparison of mRNA levels between each of the subgroups of MRL/MpJ mice and control BALB/c mice was performed using the Wilcoxon rank sum test, and the evaluation for trends over time was performed using a nonparametric test for trend. 2,7

<table>
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* Transcripts normalized to 18S rRNA.
RESULTS

Histopathology of lacrimal glands revealed little in the way of lacrimal gland inflammation in BALB/c mice at any age. MRL/H11001 mice typically had little lacrimal gland inflammation at 1.5 months of age, small foci of inflammation at 5 months of age, multiple larger foci of inflammation by 9 months of age (median, grade 3), and more extensive disease with multiple large foci of inflammation at 9 months of age (median, grade 3). MRL/lpr mice typically had lacrimal gland inflammation beginning at 1.5 months of age and extensive lacrimal gland disease with multiple inflammatory foci and lacrimal gland damage by 3 to 5 months of age (median grade 4 at both ages). The inflammation consisted primarily of mononuclear inflammatory cells (data not shown).

Results of qPCR for iNOS from mouse lacrimal glands are listed in Table 1. Both MRL/+ and MRL/lpr mice had significantly greater levels of mRNA for iNOS than did BALB/c mice. Median-normalized iNOS transcript levels were 0.34 for BALB/c mice and did not vary by age ( median, grade 3), whereas the median level for MRL/+ mice was 2.90 (p < 0.0005 vs BALB/c mice) and for MRL/lpr mice was 6.84 (p < 0.0001 vs. BALB/c mice).

Furthermore, iNOS transcript levels increased with age in both MRL/+ (p = 0.01) and MRL/lpr mice (p < 0.01). After adjustment for the different age of onset of the disease in the two substrains, there was a suggestion that MRL/lpr mice had greater levels of iNOS mRNA than did MRL/+ mice (mean 13.98 vs. 6.54, p = 0.08).

Immunohistochemistry for iNOS was performed on BALB/c, MRL/+ and MRL/lpr mouse lacrimal glands at ages 1.5, 3, and 5 months and on BALB/c and MRL/+ mice at 9 months of age. In BALB/c mice, there were a few scattered iNOS-positive mononuclear cells within the interstitial connective tissue throughout the lacrimal gland (data not shown). MRL/+ (Fig. 1) and MRL/lpr mice (Fig. 2) demonstrated scattered iNOS-positive cells but also several iNOS-positive mononuclear and dendritiform cells at the border of the inflammatory infiltrates and normal lacrimal gland tissue. Double-staining indicated that most of these cells were of the macrophage lineage (data not shown).

Results of qPCR for TNF-α from mouse lacrimal glands are listed in Table 2. In BALB/c lacrimal glands, there was a slight increase in TNF-α mRNA transcripts from 1.5 to 9 months of age (P = 0.03). TNF-α-normalized transcript levels were similar in 1.5- and 3-month MRL/+ and BALB/c mice (P = 0.98) but were greater in 5- and 9-month MRL/+ than in BALB/c mice (median 221.8 vs. 77.8, P = 0.011). There was an

FIGURE 1. Lacrimal gland from an MRL/+ mouse, (a) stained for iNOS, showing scattered positively stained cells, and (b) negative control, stained with normal goat immunoglobulin. Original magnification, ×160.

FIGURE 2. Lacrimal gland from an MRL/lpr mouse (a) stained for iNOS, showing scattered positively stained cells, and (b) negative control, stained with normal goat immunoglobulin. Original magnification, ×100.
increase in levels of TNF-α mRNA with increasing age in MRL/+ mice ($P = 0.02$). Levels of TNF-α transcripts were significantly greater in MRL/lpr mice than in BALB/c mice (median 136.7 vs. 69.2, $P = 0.001$). In MRL/lpr mice, levels of TNF-α transcripts increased with age ($P = 0.02$), consistent with the increasing lacrimal gland inflammation with increasing age in this substrain. After adjustment for the age of onset of the inflammation, there were no significant differences between MRL/+ and MRL/lpr mice (median 100.0 vs. 136.7, $P = 0.26$).

Immunohistochemistry for TNF-α was performed on BALB/c, MRL/+, and MRL/lpr mouse lacrimal glands at 1.5, 3, and 5 months of age and on BALB/c and MRL/+ mice at 9 months of age. In BALB/c mice there were a few scattered mononuclear cells staining for TNF-α throughout the lacrimal gland, largely in the interstitial connective tissue (data not shown). In MRL/+ and MRL/lpr mice there were also scattered mononuclear cells staining for TNF-α throughout the lacrimal gland in the interstitial connective tissue. In addition, in MRL/+ (Fig. 5) and MRL/lpr mice (Fig. 4) there were mononuclear and dendritiform cells staining for TNF-α at the junction of the focal inflammatory infiltrates and lacrimal gland acinar tissue. The double staining suggests that the most of the TNF-α-stained cells were also of the macrophage lineage (data not shown).

**DISCUSSION**

Both NO and TNF-α are potential mediators of tissue damage. Our results demonstrate increased levels of iNOS and TNF-α in the lacrimal glands of both MRL/+ and MRL/lpr mice compared with control BALB/c mice and increasing levels of each mediator with increasing age in both MRL/+ and MRL/lpr mice. The median levels of iNOS transcripts in the lacrimal glands of MRL/+ and MRL/lpr mice were similar to those in control BALB/c mice at 1.5 months of age, before there was lacrimal gland inflammation in MRL/+ mice and before there was much inflammation in MRL/lpr mice. However by 3 months of age the level of iNOS transcripts was 30 times greater in the lacrimal glands of MRL/+ mice than in control BALB/c mice and by 5 and 9 months it was ~50 to ~120 times greater. The levels of iNOS transcripts in the lacrimal glands of MRL/lpr mice were ~70 times greater than in BALB/c mice at 3 months of age and more than 1200 times greater at 5 months. The median level of TNF-α transcripts in the lacrimal glands of MRL/+ mice was similar to that in control BALB/c mice at 1.5 and 3 months of age but 1.9 to 3.6 times greater by 5 to 9 months of age, and the median level of TNF-α transcripts in MRL/lpr mice was 2.5 to 3.3 times greater that in BALB/c mice at all ages studied. Although the levels of TNF-α mRNA were similar in young MRL/+ and control BALB/c mice, the inflammation in MRL/+ mice did not begin until 3 months of age and an increase in TNF-α relative to BALB/c mice might be expected only in older mice. Conversely, MRL/lpr mice, which had an accelerated disease course, had inflammation at 1 month of age and had increased TNF-α levels. These results are consistent with the increasing inflammatory infiltrate in the lacrimal glands as these mice age and suggest possible roles for both NO and TNF-α in the production of tissue damage in these murine models of Sjögren’s syndrome.

Previous work by our group has suggested that lacrimal gland lesions in both MRL/+ and MRL/lpr mice may be predominantly Th2 mediated. By competitive RT-PCR we demonstrated that transcripts for IL-4 were present in 100- to 1000-fold greater amounts than were transcripts for IFN-γ and that there was a substantially increased expression of the costimulatory molecule B7-2 (CD80) over B7-1 (CD86). B7-2 induces Th2 responses, whereas B7-1 induces Th1 responses. Furthermore, in both substrains, transcripts for IL-2 and -12 were below the limit of detection, whereas IL-10 transcripts were present and increased with age. Although TNF-α typically is associated with Th1 responses, it also can be produced in Th2 responses, and Th2 responses can result in tissue damage. Experimental autoimmune uveitis (EAU) typically is a Th1-mediated process. However, in IFN-γ knockout mice, EAU becomes Th2 mediated. In the Th2-mediated model of EAU, increased levels of TNF-α are present in the ocular lesions, and thus, TNF-α has been shown to be produced both in Th1 and Th2 inflammatory responses.

NO has both regulatory and inflammatory properties. It can increase TNF-α production, may inhibit IL-2 secretion, and may increase IL-4 secretion by Th2 cells. As suggested by our iNOS results, increased levels of NO may contribute to a Th2-
mediated process in the lacrimal glands and produce tissue damage. Experiments by others in MRL/lpr mice have demonstrated increased expression of iNOS mRNA transcripts in inflamed kidneys and increased amounts of material immunoreactive for iNOS on immunohistology.11 Blocking NO production with N\(^{\text{G}}\)-monomethyl-L-arginine (NMMA), an NOS inhibitor, prevented the development of inflammatory nephritis in these mice.11 These experiments suggest that NO plays an important role in the production of tissue damage in MRL/lpr mice; however, the effect of NMMA on lacrimal and salivary glands has not been evaluated. Our data suggest that experiments aimed at blocking NO production also may be beneficial in reducing lacrimal gland inflammation in both strains.

TNF-\(\alpha\) has been detected in biopsy specimens from patients with Sjögren's syndrome.50–53 Furthermore, one uncontrolled case series suggested that treatment of patients with Sjögren's with infliximab, a monoclonal antibody to TNF-\(\alpha\), may be beneficial.54 Our data on the presence of TNF-\(\alpha\) in the lacrimal glands of both MRL/\(+\) and MRL/lpr mice suggest that these models also may mimic another aspect of the human disease.

In conclusion, our results demonstrate increased expression of both iNOS and TNF-\(\alpha\) in the lacrimal glands of MRL/\(+\) and MRL/lpr mice when compared with control BALB/c mice. Furthermore, the expression of both mediators increases with increasing age in both substrains, commensurate with the age-related increase in inflammation in the lacrimal glands. These results suggest that both iNOS and TNF-\(\alpha\) are potential mediators of tissue damage in MRL/MpJ mice. Experiments in which these molecules' production or activity are blocked should help confirm their role.

References


