

Cytokines in Autoimmune Lacrimal Gland Disease in MRL/MpJ Mice

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PURPOSE. MRL/MpJ-+/+ (MRL/+) and MRL/MpJ-*lpr/lpr* (MRL/*lpr*) mice show spontaneous development of a T-cell-driven lacrimal gland inflammation that is a model for Sjögren syndrome. The lacrimal gland lesions in these mice were evaluated by quantitative RT-PCR for selected cytokine mRNA for the relative contributions of T-helper (Th)1 versus Th2 immune responses and by RT-PCR and immunohistochemistry for the contribution of the interleukin (IL)-2/IL-2 receptor (IL-2R) autocrine pathway.

METHODS. RNA was isolated from lacrimal glands of MRL/+ mice ages 1 to 9 months and from MRL/*lpr* mice ages 1 through 5 months, and competitive RT-PCR was used to quantify mRNA for the cytokines IL-2, -4, -10, and -12 and interferon (IFN)- γ . Frozen sections of lacrimal glands from MRL/+ and MRL/*lpr* mice ages 2 through 5 months were stained for the IL-2R.

RESULTS. IL-2 and -12 mRNA transcripts were below the limit of detection ($<10^{-3}$ fg/pg hypoxanthine phosphoribosyl transferase gene; *HPRT*) in both MRL/+ and MRL/*lpr* mice of all ages. When detectable, IFN- γ transcripts were present in low amounts and were below the limit of detection in most samples. IL-4 transcripts were present in 100- to 1000-fold greater amounts than IFN- γ transcripts. IL-10 transcripts were detectable in both MRL/+ and MRL/*lpr* mice. IL-2R typically was detected on less than 10% of lymphocytes infiltrating lacrimal gland lesions in both substrains.

CONCLUSIONS. On the basis of RT-PCR for cytokine mRNA, autoimmune lacrimal gland lesions in MRL/+ and MRL/*lpr* mice appear to be largely Th2-mediated. There does not appear to be a direct role for the IL-2/IL-2R autocrine pathway within the microenvironment of the lacrimal gland. (*Invest Ophthalmol Vis Sci.* 2001;42:2567-2571)

Sjögren syndrome is among the most common autoimmune diseases in humans.¹ It is characterized by a mononuclear inflammatory cell infiltrate into the lacrimal and salivary glands, resulting in glandular damage, secretory dysfunction, and dry eyes and mouth.¹ MRL/MpJ mice show spontaneous develop-

ment of lacrimal and salivary gland inflammation and are a model for the human disorder Sjögren syndrome.²⁻⁴ There are two congenic substrains of MRL/MpJ mice, and they differ only by a single autosomal recessive mutation, the *lpr* gene.^{5,6} The *lpr* mutation results in an altered Fas protein, defective lymphocyte apoptosis, defective clonal deletion of autoreactive T cells in peripheral lymphoid organs, and defective elimination of activated T cells after response to antigen.⁷⁻⁹ Results of this defective apoptosis include accelerated autoimmune disease in MRL/MpJ-*lpr/lpr* (MRL/*lpr*) mice when compared with MRL/MpJ-+/+ (MRL/+) mice. Although inflammatory lacrimal gland lesions develop in both MRL/*lpr* and MRL/+ mice, there are differences between the two substrains. Lacrimal gland disease develops earlier in MRL/*lpr* mice than in MRL/+ mice, and at comparable ages, MRL/*lpr* mice have more severe and extensive disease.⁴ As in human Sjögren syndrome,^{10,11} the lacrimal gland lesions in both substrains of MRL/MpJ mice are composed largely of T cells (approximately 80%), the majority of which are CD4⁺ T cells. Lesser numbers of CD8⁺ T cells, B cells, and macrophages are present. MRL/*lpr* mice typically die at 6 months of age, whereas MRL/+ mice often live to 2 years of age. In aged (18 months) MRL/+ mice there is an accumulation of B cells in the lacrimal gland lesions.^{3,4,12}

CD4⁺ helper T (Th) cells differentiate through two pathways into Th1 or Th2 cells and have different effector mechanisms.¹³⁻¹⁹ Th1 cells produce interferon (IFN)- γ and tumor necrosis factor and are primarily responsible for cell-mediated immune responses, including delayed-type hypersensitivity. Th2 cells produce interleukin (IL)-4, -5, and -10 and provide help for B cells in antibody production in humoral immune responses. Cytokines are involved in directing the immune responses toward a Th1 or Th2 type. IL-12 and IFN- γ production leads to Th1 responses, whereas IL-4 results in Th2 responses and IL-10 inhibits Th1 responses.¹⁴⁻¹⁹ The IL-2/IL-2 receptor (IL-2R) autocrine pathway is involved in the expansion of the immune response, particularly in cell-mediated immune responses.

To better understand the pathogenesis of autoimmune lacrimal gland disease in MRL/MpJ mice, we evaluated cytokine mRNA transcripts related to Th1 and Th2 responses and evaluated the IL-2/IL2R autocrine pathway in the lacrimal gland lesions of both substrains.

MATERIALS AND METHODS

Animals

MRL/MpJ mice of both substrains were obtained from the Jackson Laboratories (Bar Harbor, ME) at 1 month of age and kept under standard conditions until killed. Mice of each strain were anesthetized and killed by exsanguination in numbers and at the ages to be detailed later in the article. At the time of death, lacrimal glands were removed and processed either for reverse transcription-polymerase chain reaction (RT-PCR) for mRNA for selected cytokines or for immunohistochemistry. These experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by The Johns Hopkins Medical Institutions Animal Care and Use Committee.

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PQRS Plasmid Preparation

Plasmid PQRS (a kind gift of Ed Wakil, David B. Corry, and Linda Stowing, University of California, San Francisco²⁰) was used to transform *Escherichia coli*, and the plasmid was prepared by standard methods.^{20,24} PQRS was liberated from the plasmid by digestion with *NotI* and *SfiI*. The 4.1-kb band was obtained from a 1.4% agarose gel.

Competitive RT-PCR for Cytokines

Total nucleic acids were extracted from the lacrimal glands of MRL/MpJ mice of both substrains at selected ages with RNA extraction reagent (Trizol; Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions.²¹⁻²⁴ Samples represented pooled lacrimal glands from both glands in each mouse. After chloroformisoamyl alcohol extraction and ethanol precipitations, samples were DNaseI treated and re-extracted, with further ethanol precipitation. RNA concentrations were determined for each sample by spectrophotometry. Samples were confirmed to be DNA-free by PCR without reverse transcription targeting of the hypoxanthine phosphoribosyl transferase (*HPRT*) gene (described later).

RNA at 2 to 4 μ g per sample was used as the template for reverse transcription, as previously described,²¹⁻²⁴ with the murine leukemia virus (MuLV) enzyme (Life Technologies) and random hexamers as primers, according to the manufacturer's instructions. After reverse transcription, each sample was treated with RNase H and A (3 μ l/tube) by incubation at 37°C for 30 minutes followed by 94°C for 3 minutes. Samples were re-extracted with phenol:chloroform, and the cDNA ethanol precipitated. Samples were stored at -70°C until PCR was performed.

Hypoxanthine phosphoribosyl transferase, a housekeeping gene, and cytokine primers used for these competitive PCR studies were those published by Reiner et al.²⁰ and were synthesized by Life Technologies. Competitive PCR was performed with the first-strand cDNA using the methods of Reiner et al.²⁰ Initially, each sample was amplified without competitor plasmid and targeting *HPRT* to guide subsequent sample and competitor inputs for the quantitative assays. The quantitative competitor PCR was run in three duplicate tubes, each of which received one of three known concentrations of the competitor PQRS plasmid DNA; each final sample volume was brought to 50 μ l with deionized distilled water (UltraPure; Life Technologies); and PCR was performed on a thermocycler (MJ Research, Waltham, MA) programmed as recommended by Reiner et al.²⁰ Amplification products were visualized on 1.5% agarose gels after ethidium bromide staining. Digitized photographs were taken for analysis of *HPRT*, IFN- γ , and IL-2, -4, -10, and -12 cDNA amplification products, and quantitation was achieved on a fluorescence imager (Hewlett Packard, Palo Alto, CA). Cytokine transcript levels determined by known input pQRS concentrations were normalized to 1 pg *HPRT*. Thymus was used as a source of normal tissue and as a positive cytokine control. BALB/c lacrimal glands served as a negative control for cytokines and were negative except for *HPRT* amplification products (data not shown). Results are expressed as femtograms mRNA cytokine transcripts per picogram *HPRT* mRNA. The lower limit of detection for this assay was 1×10^{-3} fg/pg *HPRT*.

Immunohistochemistry

Lacrimal glands processed for immunocytochemistry were removed, embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN), frozen in liquid nitrogen, and sectioned at 8 μ m on a cryostat. Staining of frozen sections of lacrimal glands for the IL-2R was performed using a rat monoclonal antibody to the IL-2R (PharMingen, San Diego, CA) and the avidin-biotin-peroxidase complex (ABC) technique.^{3,4,12,25,26} Frozen sections were fixed in chilled (4°C) acetone, air dried, rehydrated in phosphate-buffered saline (PBS), and incubated with the appropriate blocking agent (Vector, Burlingame, CA) for 15 minutes. The primary antibody was applied, and the slides were incubated for 60 minutes. A second blocking step was then performed. The slides were washed in PBS, incubated with biotinylated secondary

TABLE 1. Cytokine mRNA Transcripts in Lacrimal Glands in MRL/MpJ Mice

Age (mo)	n	IFN- γ	IL-4	IL-10
MRL/MpJ+/+ Mice				
1	4	0.005 \pm 0.008	1.5 \pm 1.8	0.24 \pm 0.21
3	3	0.003 \pm 0.004	3.8 \pm 1.5	2.4 \pm 2.3
5	5	0.003 \pm 0.068	7.1 \pm 2.0	4.9 \pm 2.4
9	2	0.02 \pm 0.02	11.5 \pm 2.5	3.9 \pm 0.6
MRL/MpJ-lpr/lpr Mice				
1	3	Undetectable	3.3 \pm 4.4	0.07 \pm 0.08
3	3	0.01 \pm 0.01	23 \pm 3.6	2.3 \pm 1.2
5	4	0.04 \pm 0.06	53 \pm 19	8.0 \pm 2.1

Data are mean mRNA transcripts \pm SEM expressed in femtograms per picogram *HPRT* mRNA. IL-2 and -12 transcripts were undetectable in competitive RT-PCR ($< 1 \times 10^{-3}$ fg/pg *HPRT*).

antibody for 30 minutes, rinsed in PBS, incubated with the ABC reagent (ABC-Elite Kit; Vector) for 45 minutes, washed again in PBS, developed with 3% hydrogen peroxide, and 3-amino-9-ethyl-carbazole-containing acetate buffer, and counterstained with Harris's hematoxylin (Sigma, St. Louis, MO). The percentage of mononuclear inflammatory cells staining positively was enumerated using a 10×10 - μ m grid disc that covered a 0.16-mm² area using a $\times 25$ objective and a $\times 10$ ocular mounted on a standard binocular microscope (Carl Zeiss, Oberkochen, Germany).^{3,4,13,25} For each staining run, appropriate positive control (spleen sections) and negative control experiments, in which normal rat immunoglobulin was substituted for the primary antibody, were performed for quality control.

Statistics

The comparison of the levels of mRNA transcripts between IL-4 and IFN- γ was performed using the sign test, a nonparametric paired analysis. The evaluation of trends over time for cytokines within a substrain was performed using linear regression; the results are expressed as β , the slope of the regression line. The comparison between substrains was performed using multiple linear regression.²⁷

RESULTS

The amounts of mRNA for selected cytokines transcribed per pair of lacrimal glands are shown as Table 1. IL-2 mRNA transcripts were below the limit of detection at all time points in both MRL/+ or MRL/lpr mice. IFN- γ transcripts, when detectable, were present in low amounts but were below the limit of detection in 70% of samples. The amounts of IL-4 transcripts were 100 to 1000 times greater than those of IFN- γ in both MRL/+ ($P < 0.001$) and MRL/lpr ($P = 0.002$) mice at all time points. In both MRL/+ and MRL/lpr mice there was an increase in IL-4 transcripts over time, consistent with the increasing proportion of the lacrimal gland replaced by inflammatory infiltrate.⁴ The slopes of linear regression were 1.28 ± 0.22 fg IL-4 mRNA/pg *HPRT* mRNA/month ($P < 0.001$) for MRL/+ mice and 12.60 ± 2.74 fg IL-4 mRNA/pg *HPRT* mRNA/month ($P = 0.002$) for MRL/lpr mice. At comparable ages, MRL/lpr mice had greater amounts of IL-4 transcripts than did MRL/+ mice ($P < 0.001$), consistent with the greater proportion of lacrimal gland replaced by the inflammatory infiltrate in MRL/lpr mice.⁴

IL-10 transcripts were detected in both MRL/+ and MRL/lpr mice. In both MRL/+ and MRL/lpr mice there was a progressive increase in IL-10 transcripts over time. The slopes of the linear regression for IL-10 were 2.09 ± 0.65 fg IL-10 mRNA/pg *HPRT* mRNA/month ($P = 0.007$) for MRL/+ mice and 2.00 ± 0.35 fg IL-10 mRNA/pg *HPRT* mRNA/month ($P < 0.001$) for MRL/lpr mice. There were no significant differences between

the two substrains in the amount of IL-10 transcripts ($P = 0.08$); however, the number of samples studied was small. IL-12 transcripts were below the limit of detection at all time points in both substrains. The 100- to 1000-fold greater amounts of IL-4 transcripts than IFN- γ transcripts and the ability to detect IL-10 transcripts but not IL-12 transcripts all are consistent with a Th2-mediated inflammatory process in the autoimmune lacrimal gland disease of both substrains.

IL-2 receptor expression is shown as Table 2. One-month-old animals have insufficient inflammatory infiltrates for immunohistochemistry. Only one of the five 2-month-old MRL/+ mice had an inflammatory infiltrate in the lacrimal gland. Between 3 and 5 months of age the proportion of cells staining for the IL-2R was low in MRL/+ mice (Fig. 1), although there appeared to be an increase over time in the proportion of cells staining for IL-2R. In MRL/lpr mice, there was a low and relatively unchanged proportion of cells staining for the IL-2R (Fig. 1). The relatively small proportion of cells staining positively for IL-2R and the inability to detect IL-2 transcripts both are consistent with a limited direct role for the IL-2/IL-2R autocrine pathway in lacrimal gland inflammation in MRL/+ and MRL/lpr mice.

DISCUSSION

In both the human disorder Sjögren syndrome and the murine model for Sjögren syndrome, the MRL/MpJ mouse, most cells in the inflammatory infiltrate are CD4⁺ T cells.^{3,4,10-12} Using immunohistochemistry, we previously reported that a majority of the CD4⁺ cells in the lacrimal gland lesions of both MRL/+ and MRL/lpr mice stained for IL-4 and that only a small number stained for IFN- γ .²⁵ In addition, we reported a greater expression of the costimulatory molecule B7-2 than of B7-1 on antigen-presenting cells in the lacrimal glands of both substrains of MRL/MpJ mice.²⁵ B7 is a costimulatory molecule expressed on antigen-presenting cells and is required for the effective stimulation of T cells to respond to antigen presentation; it exists as two major subtypes, B7-1 and B7-2. B7-1 is the costimulatory molecule for Th1 responses and B7-2 is the costimulatory molecule for Th2 responses.^{28,29} Our previous results were consistent with a Th2-mediated process in the lacrimal gland lesions of both substrains of MRL/MpJ mice.²⁵

Because Th1 responses sometimes are labeled "proinflammatory" and Th2 responses "regulatory" these results were somewhat surprising. Therefore, we evaluated IL-4 and IFN- γ in lacrimal gland disease using an alternative method (competitive RT-PCR), evaluated additional cytokines (IL-10 and -12), and evaluated the IL-2/IL-2R autocrine pathway. Using a different assay technique, competitive RT-PCR, we demonstrated more than 100 times greater levels of IL-4 mRNA than of IFN- γ .

TABLE 2. IL-2 Receptor Expression in Lacrimal Glands in MRL/MpJ Mice

Age (mo)	<i>n</i>	Mice with lesions (<i>n</i>)	Positive for IL-2R*
MRL/MpJ-+/+ Mice			
2	5	1	13
3	5	4	3.2 ± 1.1
4	4	4	5.5 ± 2.2
5	4	4	10.8 ± 3.3
MRL/MpJ-lpr/lpr Mice			
2	5	4	8.4 ± 6.1
3	5	5	8.2 ± 1.8
4	5	5	5.2 ± 1.5
5	5	5	8.2 ± 5.0

* Mean percentage ± SEM.

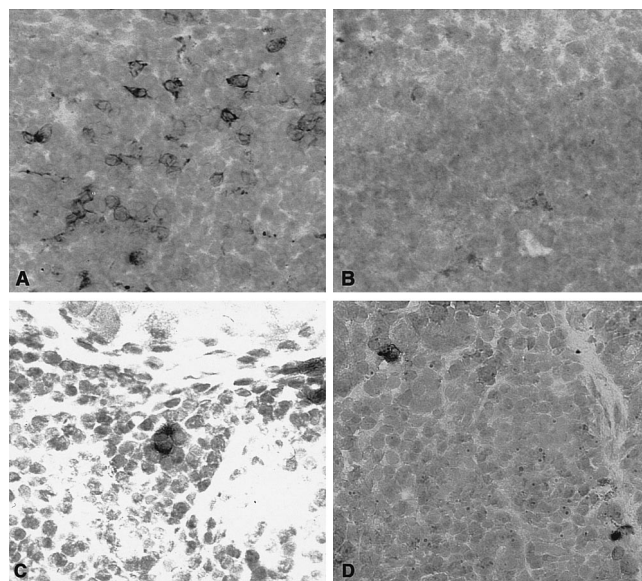


FIGURE 1. Staining for the interleukin-2 receptor. (A) Positive control spleen section from a BALB/c mouse. (B) Negative control spleen section, omitting primary antibody. (C) Lacrimal gland section from a 5-month-old MRL/+ mouse and (D) lacrimal gland section from a 3-month-old MRL/lpr mouse showing a paucity of cells staining positively in both sections. Original magnification, (A, B) ×125; (C, D) ×165.

These results are consistent with a Th2-mediated process in the lacrimal glands of both MRL/+ and MRL/lpr mice. In addition, IL-10 mRNA transcripts were detected, whereas IL-12 transcripts were not. IL-12 is produced by antigen-presenting cells and known to drive the immune response toward Th1 responses, whereas IL-10 inhibits Th1 responses.^{30,31} These results further support the hypothesis that the lacrimal gland lesions in both substrains of MRL/MpJ mice are Th2 mediated.

The effects of blocking B7-1 and B7-2 costimulation with monoclonal antibodies or using B7-1- or B7-2- deficient mice (knockout mice) have been evaluated in the MRL/lpr substrain. MRL/lpr deficient in B7-1 or B7-2 still produce autoantibodies, but the absence of B7-2 costimulation interferes with the spontaneous activation and accumulation of memory CD4⁺ and CD8⁺ T lymphocytes. In addition, B7-2- deficient MRL/lpr mice have lesser degrees of glomerulonephritis than do wild-type mice, whereas B7-1- deficient MRL/lpr have more severe glomerulonephritis than do wild type MRL/lpr mice.³² Although lacrimal and salivary gland lesions were not evaluated in this study, these data suggest that Th2 responses play a role in disease pathogenesis in MRL/lpr mice. In addition, C57BL/6 mice made transgenic for the IL-10 gene regulated by the salivary amylase promoter showed development of lacrimal and salivary gland exocrinopathy, in which the lymphocyte infiltration into the glands consisted primarily of CD4⁺ T cells.³³ These results suggest that IL-10, either directly or by suppression of Th1 responses, may promote the development of autoimmune lacrimal gland disease.

Although the evaluation of minor salivary gland biopsy specimens from patients with Sjögren syndrome has produced various results for the cytokines detected,³⁴⁻³⁶ Aziz et al.³⁶ reported that IL-4 mRNA was detected in 33% of the infiltrating mononuclear inflammatory cells, compared with IFN- γ detected in only 13%, and that only IL-4 mRNA positive cells were detected in a statistically significant excess over control biopsy specimens. They concluded that a Th2 process was present in the glandular inflammatory infiltrate in patients with Sjögren syndrome, results similar to those in MRL/MpJ mice.

MRL/lpr mice have an accelerated autoimmune disease when compared with MRL/+ mice and show development of lacrimal gland inflammation at an earlier age than do MRL/+ mice.⁴ The onset of lacrimal gland disease in MRL/lpr mice is typically at 1 month of age, whereas in MRL/+ mice it is at 3 months. At comparable ages, MRL/lpr mice have more extensive disease, both in severity and extent of lacrimal gland inflammation. Although MRL/lpr mice typically die by 6 months of age, MRL/+ mice have a normal life span. The *lpr* gene induces a defective Fas protein resulting in defective lymphocyte apoptosis, occurring primarily in peripheral lymphoid organs.⁷⁻⁹ This defect results in an accumulation of autoreactive lymphocytes, massive lymphadenopathy, and the accelerated autoimmune disease present in MRL/lpr mice. Using a nonquantitative RT-PCR assay, Murray et al.³⁷ and Takahashi et al.³⁸ have reported that the autoimmune mechanism in MRL/lpr mice spleens and lymph nodes appears to be Th1 in nature.

In addition, spleens and lymph nodes of MRL/lpr, but not MRL/+, mice are infiltrated by CD3⁺, CD4⁻, CD8⁻, B220⁺, and TCR α/β ⁺ double-negative T cells.³⁹ However, evaluation of lymph node and spleen cells from other mice homozygous for the *lpr* gene has demonstrated that the double-negative T cells do not secrete the cytokines IFN- γ and TNF- α ; CD4⁺ T cells secrete these cytokines, and they are produced in greater levels than are cytokines from similar strains without the *lpr* mutation.⁴⁰ Our previous studies suggest that double-negative T cells do not have a role in lacrimal gland disease in MRL/lpr mice. Selective depletion of CD4⁺ and CD8⁺ T cells with monoclonal antibodies (which would not affect double-negative T cells) eliminates the lacrimal gland disease in MRL/lpr mice.⁴¹ CD4⁺ T cells play a key, but not an exclusive, role in the lacrimal gland disease in MRL/lpr mice and a key role in the systemic autoimmunity in MRL/lpr mice.⁴¹⁻⁴³ Hence, CD4⁺ T cells isolated from sialadenitis lesions in MRL/lpr mice transfer lacrimal and salivary gland disease to SCID mice.

Because our data show that the CD4⁺ T cells in the glandular lesions are largely Th2 in nature, it suggests that the disease is transferred by the Th2 cells. Furthermore, the lacrimal gland disease in MRL/lpr mice appears to be mediated in a different fashion than is the systemic autoimmune disease.⁴² MRL/lpr and MRL/+ mice have a similar immunopathogenic mechanism for lacrimal gland disease, and MRL/+ mice do not have the systemic autoimmunity engendered by the *lpr* gene. Therefore, it appears that the lacrimal gland disease is intrinsic to the MRL/Mp strain and has a pathogenic mechanism that, at least in part, may be different than that seen in the systemic autoimmunity of MRL/lpr mice.

MRL/lpr lymphoid tissues have a normal IL-2/IL-2R autocrine pathway at young ages, but have defects in both the production of and response to IL-2 after 3 months of age.^{45,46} Conversely, MRL/+ mice appear to be comparatively normal in the IL-2/IL-2R autocrine pathway throughout their life spans. The failure to detect IL-2 mRNA and the low proportion of cells staining positively for the IL-2R in MRL/MpJ mice suggest that the IL-2/IL-2R autocrine pathway does not play a direct role in the autoimmune disease within the microenvironment of the lacrimal gland in both MRL/+ and MRL/lpr mice. Although defects in the MRL/lpr IL-2 response may account for this result in MRL/lpr mice at 3 months or more of age, the absence of such defects in the systemic IL-2/IL-2R pathway in MRL/+ mice implies that the autoimmune process in the lacrimal gland of MRL/MpJ mice does not use the IL-2/IL-2R pathway to any substantial degree.

Of interest, mice without the IL-2R β chain have spontaneously activated CD4⁺ T cells, resulting in differentiation of B cells into plasma cells, high serum concentrations of immunoglobulin, and autoantibody formation.⁴⁷ These data suggest

that the IL-2R is required to keep the activation programs of T cells under control to maintain homeostasis and prevent autoimmunity. Hence, dysregulation of the IL-2/IL-2R system in the lacrimal gland lesions of MRL/MpJ mice may contribute to the autoimmune disease and to the Th2 pathogenesis of these lesions.

Although the evaluation of minor salivary gland biopsy specimens from patients with Sjögren syndrome also has given variable results for the presence of IL-2 mRNA,³⁴⁻³⁶ Aziz et al.³⁶ detected little in the way of IL-2R expression. Thus, MRL/MpJ mice lacrimal gland results may be similar to those in minor salivary glands from patients with Sjögren syndrome, and the IL-2/IL-2R pathway may not play a direct role in the tissue damage in the lacrimal gland autoimmune disease in patients with Sjögren syndrome.

In conclusion, our results using competitive RT-PCR to detect mRNA for cytokines suggest that the lacrimal gland lesions in both substrains of MRL/MpJ mice are Th2 mediated. Furthermore, our RT-PCR results for IL-2 and immunohistochemistry results for the IL-2R suggest a limited role for the IL-2/IL-2R autocrine pathway in the production of lacrimal gland inflammation in both MRL/+ and MRL/lpr mice. However, overproduction of IL-10 and downregulation of the IL-2/IL-2R pathway may contribute to the immune dysregulation in the lacrimal glands and to the production of a Th2-mediated disease.

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