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High incidence of autoimmune dacryoadenitis in male non-obese diabetic (NOD) mice depending on sex steroid

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SUMMARY

The NOD mouse develops spontaneous autoimmune lesions in the lacrimal and salivary glands, besides a well characterized T cell-mediated autoimmune pancreatic β cell lesion. We report unique pathological findings developed in the lacrimal glands as an autoimmune dacryoadenitis of NOD mice in contrast to those found in the salivary glands and pancreas. A high incidence of autoimmune lesions in the lacrimal glands was observed exclusively in male NOD mice at any age. Histology of autoimmune dacryoadenitis in male NOD mice showed severe destructive changes compared with those observed previously as an autoimmune lesion in the lacrimal glands. Castration in male NOD mice significantly decreased the incidence of autoimmune dacryoadenitis, and testosterone treatment with castration also increased the incidence of autoimmune lesions. Oestrogen treatment with castration did not increase the incidence, but tamoxifen treatment without castration significantly increased the incidence of autoimmune dacryoadenitis in NOD mice. In addition, we detected up-regulation of local cytokine genes (IL-1β, tumour necrosis factor-alpha (TNF-α), IL-2, interferon-gamma (IFN-γ), IL-6, IL-10, and IL-12 p40) during the course of autoimmune dacryoadenitis. These data suggest that in spontaneous autoimmune dacryoadenitis of male NOD mice there may be an intimate relationship with sex steroids, particularly testosterone, in the development and progression of autoimmune lesions, and autoreactive Th1 cells secrete up-regulated cytokine genes, including IL-10 and IL-12.

Keywords Sjögren's syndrome lacrimal gland cytokine sex steroid NOD mice

INTRODUCTION

It is well known that sex steroids have significant impact on the development and progression of human and experimental autoimmune disease. Sex steroids have been suggested to be responsible for the strong female preponderance of human systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS) [1–4]. Testosterones and oestrogens have important roles in the development of autoimmune lesions, and they also modulate immune responses in a number of animal models [5–7]. Insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated, organ-specific autoimmune disease that occurs in humans and in an animal model such as the NOD mouse [8,9]. Autoimmune diabetes in NOD mice is characterized by lymphocytic infiltration in the islets (insulitis) followed by the destruction of islet β cells. The mononuclear cell infiltrate in NOD insulitis is mostly composed of $CD4^+$ Th1 cells [10]. It is well characterized that the immune response is more active in females than in males [6], and it has been demonstrated that testosterone administration decreased the appearance of autoimmune diabetes, while oestrogen injections increased the incidence of diabetes in NOD mice [11]. Since testosterone is a potent determinant of development, it may establish a physiological role that leads to the sexual dimorphism in the development of autoimmune diabetes in NOD mice [12]. NOD mice display inflammatory infiltration of other organs, among which the salivary glands are most often affected [13–15]. NOD sialadenitis is assumed to be similar to those occurring in various animals models of SS [16–18]. However, spontaneously occurring autoimmune lesions in the lacrimal glands of NOD mice have not yet been analysed. Several animal models for investigating autoimmune dacryoadenitis are known to develop in autoimmune-prone mice [19–21], and in non-autoimmune-prone mice [22–24]. We found the strong male preponderance of autoimmune dacryoadenitis in NOD mice similar histologically to that occurring in human SS [25]. The aim of this study was to analyse the

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natural course of autoimmune dacryoadenitis in NOD mice, the effect of sex steroids on the development and progression of autoimmune lesions in lacrimal glands, and to examine the *in vivo* gene expression of various cytokines during the disease process.

MATERIALS AND METHODS

Mice and experimental design

Male and female NOD mice were bred in our own facilities, maintained in a specific pathogen-free mouse colony and given food and water *ad libitum*. In this study, a total of 119 NOD mice, consisting of 69 male and 50 female mice, was investigated. They were killed by cervical dislocation subsequently during the time intervals ranging from 4, 8, 10, 12, 16, 20, 24 to 30 weeks. For normal controls, 20 female BALB/c mice were used, and killed at 4, 8, 12 and 20 weeks.

Experimental manipulation of sex steroid levels

Male NOD mice were manipulated to examine the effect of castration, and castration plus administration of sex steroids (testosterone and oestrogen) and non-steroidal anti-oestrogen tamoxifen. Castration of male NOD mice was performed at 4-weeks-old ($n = 22$). Mice with castration alone ($n = 7$) and mice with castration plus administration of testosterone ($n = 5$), oestrogen ($n = 5$), and tamoxifen ($n = 5$) were histologically examined at 8-weeks-old. Castrated males received every other day subcutaneous injections of testosterone in olive oil (Wako Pure Chemical Inc., Ltd, Osaka, Japan) in 25 mg/kg per day. Oestrogen dissolved in sesame oil (Ovahormone depot, Teikoku Zoki, Japan) was injected once a week subcutaneously in 60 mg/kg per week, and the non-steroidal anti-oestrogen tamoxifen was administrated every other day with subcutaneous injections of 2·5 mg/kg per day.

Histology and immunohistochemistry

All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde pH 7·2 and prepared for histological examination. The sections were stained with haematoxylin and eosin (H–E). Histological grading of the inflammatory lesions was done according to the method proposed by White & Casarett [26]. Immunohistochemical staining with MoAbs was performed on freshly frozen sections using the avidin-biotin immunoperoxidase method. Briefly, freshly frozen sections were fixed in acetone for 10 min, rinsed in PBS pH 7·2, and incubated with an appropriate blocking agent (Vector Labs, Inc., Burlingame, CA) for 20 min. They were incubated for 1 h with the first MoAbs as follows: biotinylated rat MoAbs to CD3 (Life Technologies, Grand Island, NY), B220, L3T4 (CD4), Lyt2 (CD8), and Mac-1 (Becton Dickinson, San Jose, CA). Commercially available MoAbs to mouse cytokines used were: anti-IL-2, -IL-4, -IL-6, -interferon-gamma (IFN-γ), -tumour necrosis factor-alpha (TNF-α) (Pharmingen, San Diego, CA). Rat IgM MoAb to IL-10 (SXC.1) was kindly provided from Dr Hiroshi Ishida (Department of Medicine, Utano National Hospital, Kyoto, Japan). Anti-rat IgG and IgM sera (Vector Labs) were used as the second antibodies for 30 min after the first incubation. Sections were washed with cold PBS for 30 min and incubated with ABC complex (Vector Labs) for 30 min. After washing with PBS, sections were reacted with a fresh mixture of 0·05% 3,3′-diaminobenzidine and 0·005% H_2O_2 in Tris buffer (0·05 M, pH 7·6) for 5 min, washed with distilled water, and counterstained with methyl green. All control samples treated with normal rat and hamster sera (Cappel Labs, Cochranville, PA) or PBS instead of the first antibodies gave negative results.

Reverse transcriptase-polymerase chain reaction and Southern blot analysis

To analyse gene expressions of various cytokines by reverse transcriptase-polymerase chain reaction (RT-PCR) method, total RNA prepared from the lacrimal glands, and spleens was reverse transcribed into complementary DNA (cDNA). Total RNA preparation was performed essentially as reported previously [27]. Briefly, aliquots of cells or tissues were lysed in 200 μl of ice-cold lysis solution D, which contained 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7·0, 0·1 M 2-mercaptoethanol (2-ME), and 0·05% (w/v) sarcosyl. The mixture was chilled on ice and centrifuged at 10 000 ***g*** for 20 min at 4°C, and the supernatant was treated with an equal volume of isopropanol at −20°C for 90 min. The mixture was centrifuged and the pellets were incubated with solution D at 20°C for 60 min. The RNA was washed with 75% ethanol in diethylpyrocarbonate (Aldrich Chemical Co., Milwaukee, WI)-treated water, centrifuged, and stored at −80°C until further processing. For analysis of cytokines by RT-PCR, RNA was reverse transcribed into cDNA. The reaction mixture was added to the RNA solution and incubated at 42°C for 30 min. Then it was heated at 94°C for 5 min and chilled on ice. For PCR, the cDNA reaction mixture was diluted with 90 μl of PCR buffer and mixed with 50 pmol of the 5′ and 3′ primer, 1·25 mM dNTP, 20 mM $MgCl_2$, and 2 U of thermostable Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Evaporation was prevented by addition of 150 μl of mineral oil and the reaction was carried out by denaturing the RNA–cDNA hybrid by heating at 94°C for 30 s, annealing the primers at 55°C for 30 s, and extending the primers at 72°C for 1 min. This cycle was repeated 35 times in a DNA thermal cycler (Perkin Elmer Cetus). After the final cycle, the temperature was maintained at 72°C for 10 min to allow re-annealing of the amplified products, and the mixture was then chilled. A 10-μl aliquot of the amplified DNA reaction mixture was subjected 1·7% agarose gel electrophoresis, and the amplified product was visualized by UV fluorescence after staining with ethidium bromide. PCR products were blotted onto nylon membrane and hybridized with ^{32}P-labelled cDNA probes. The primers used were synthesized by the phosphoamide method in a DNA synthesizer (model 391 PCR-MATE; Applied Biosystems, Foster City, CA) and purified on Sephadex G50 columns (Pharmacia LKB, Piscataway, NJ) by high performance liquid chromatography (HPLC). The sequences of the primers were specific, as confirmed by a computer search of updated versions of GenBank, and were chosen to have a balanced nucleotide composition with a G-C content of 40–60%. The cDNA was subjected to enzymatic amplification in a DNA thermal cycler (Perkin Elmer Cetus) by using specific primers as shown in Table 1.

RESULTS

Histopathological findings

Male NOD mice with autoimmune dacryoadenitis develop early onset, and have severe inflammatory lesions at all ages, compared with autoimmune sialadenitis and insulitis in female NOD mice. Histology of autoimmune dacryoadenitis in male NOD mice shows destructive changes with inflammatory infiltration, and the severity overcomes the previously observed autoimmune lesions in the lacrimal glands [19–24]. Figure 1 shows a summary of the mean

Table 1. Primer sequences for reverse transcriptase-polymerase chain reaction*

Gene			Sequence
Cytokines:	IL1β,	5′	TGATGAGAATGACCTGTTCT
		3′	CTTCTTCAAAGATGAAGGAAA
	TNF-α	5′	ATGAGCACAGAAAGCATGATC
		3′	AGATGATCTGAGTGTGAGGG
	IFN-γ	5′	CCTCAGACTCTTTGAAGTCT
		3′	CAGCGACTCCTTTTCCGCTT
	IL-2	5′	GCAGGATGGAGAATTACAGG
		3′	GTTGTCAGAGCCCTTTAGTTT
	IL-4	5′	CACTTGAGAGAGATCATCGG
		3′	GGCTTTCCAGGAAGTCTTTCA
	IL-6	5′	CTCTGCAAGAGAGACTTCCAT
		3′	ATAGGCAAATTTCCTGATTATA
	IL-10	5′	CAGCCAGGTGACTTTC
		3′	TTCACAGGGGAGAAATCGAT
	IL-12	5′	GTTTTGCTGTCTCCACA
		3′	TCTACTTCAAGTCCATGTTTCT
β-actin:		5′	GTGGGCCGCTCTAGGCACCA
		3′	CGGTTGGCCTTAGGGTTCAGGGGG
	Cβ	5′	ATGTGACTCCACCCAAGGTCTCCTTGTTTG
		3′	TTGCAGACAGAACCCCCTGATGATAGGATG

*All sequences were obtained from GenBank.
TNF-α, Tumour necrosis factor-alpha.

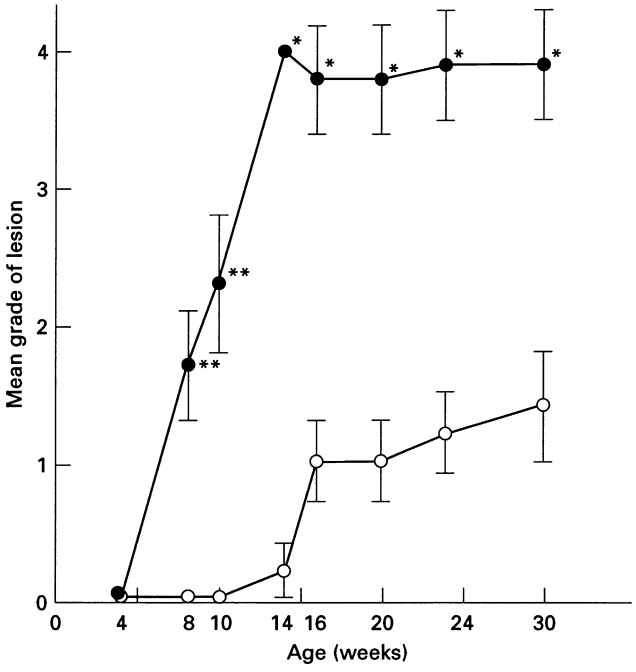

Fig. 1. Mean grade of inflammatory lesions of the lacrimal glands developing in male (●) and female (○) NOD mice. Grading of the inflammatory lesions was classified according to the methods of White & Casarett [26]. Data represent the mean grade of lesions ± s.d. (Mann–Whitney *U*-test). *$P < 0{\cdot}01$; **$P < 0{\cdot}05$.

grade of inflammatory lesions in the lacrimal glands of NOD mice. A significantly higher incidence of autoimmune dacryoadenitis in males than in females was observed at all ages. No significant inflammatory changes were found in the lacrimal glands of female NOD mice until 30 weeks of age. Figure 2 A,B show the representative histological features of autoimmune dacryoadenitis in male NOD mice.

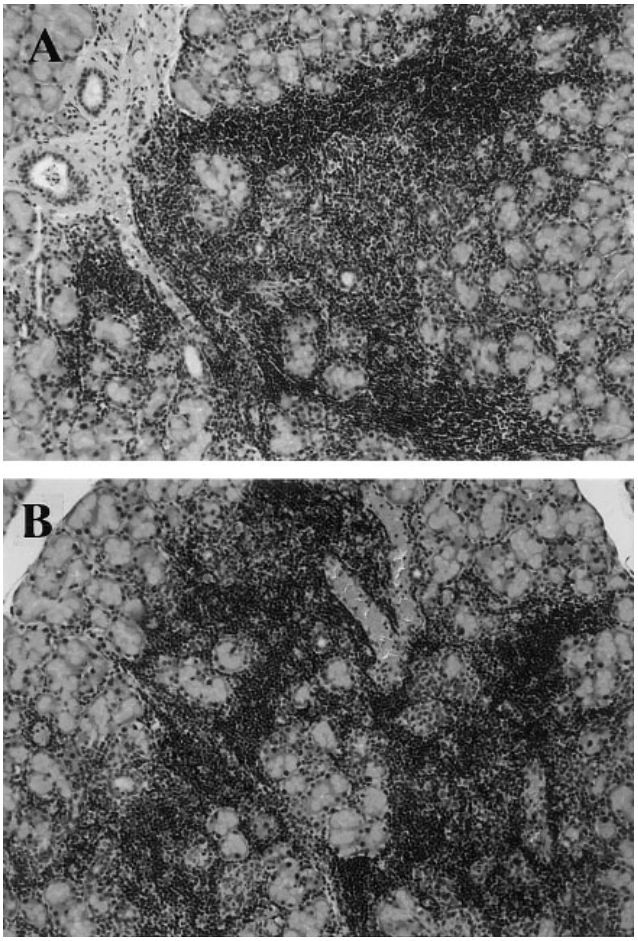

Fig. 2. Histology of inflammatory lesions in the lacrimal glands of male NOD mice. Severe, destructive inflammatory infiltrates (grade 4) were observed at 14 weeks old (A), and 30 weeks old (B). (Haematoxylin and eosin, ×120.)

Immunohistochemical findings

Immunohistochemical analysis to identify cell populations revealed that a major proportion of infiltrating cells was $CD3^+$ and $CD4^+$ (Fig. 3A), with a small number of $CD8^+$ and $B220^+$ cells from the onset of disease. Mac-1^+ mononuclear cells were observed, but sporadically within the inflammatory lesions. Immunohistochemical analysis using the ABC method was carried out to identify the particular cells responsible for production of various kinds of cytokines (TNF-α, IL-2, IFN-γ, IL-4, IL-6 and IL-10) in the lacrimal glands of male NOD mice. A variable proportion of cytokine-positive cells (TNF-α, IL-2, IFN-γ, IL-6 and IL-10) was frequently detected in the lacrimal glands from the onset of inflammatory lesions at 10 weeks old, and their numbers increased with age until 30 weeks old. IL-4^+ cells were detected later in the inflammatory lesions. Figure 3B–D show the representative immunohistochemical features of the infiltrating cells positive for cytokines in the lacrimal glands of 14-week-old male NOD mice.

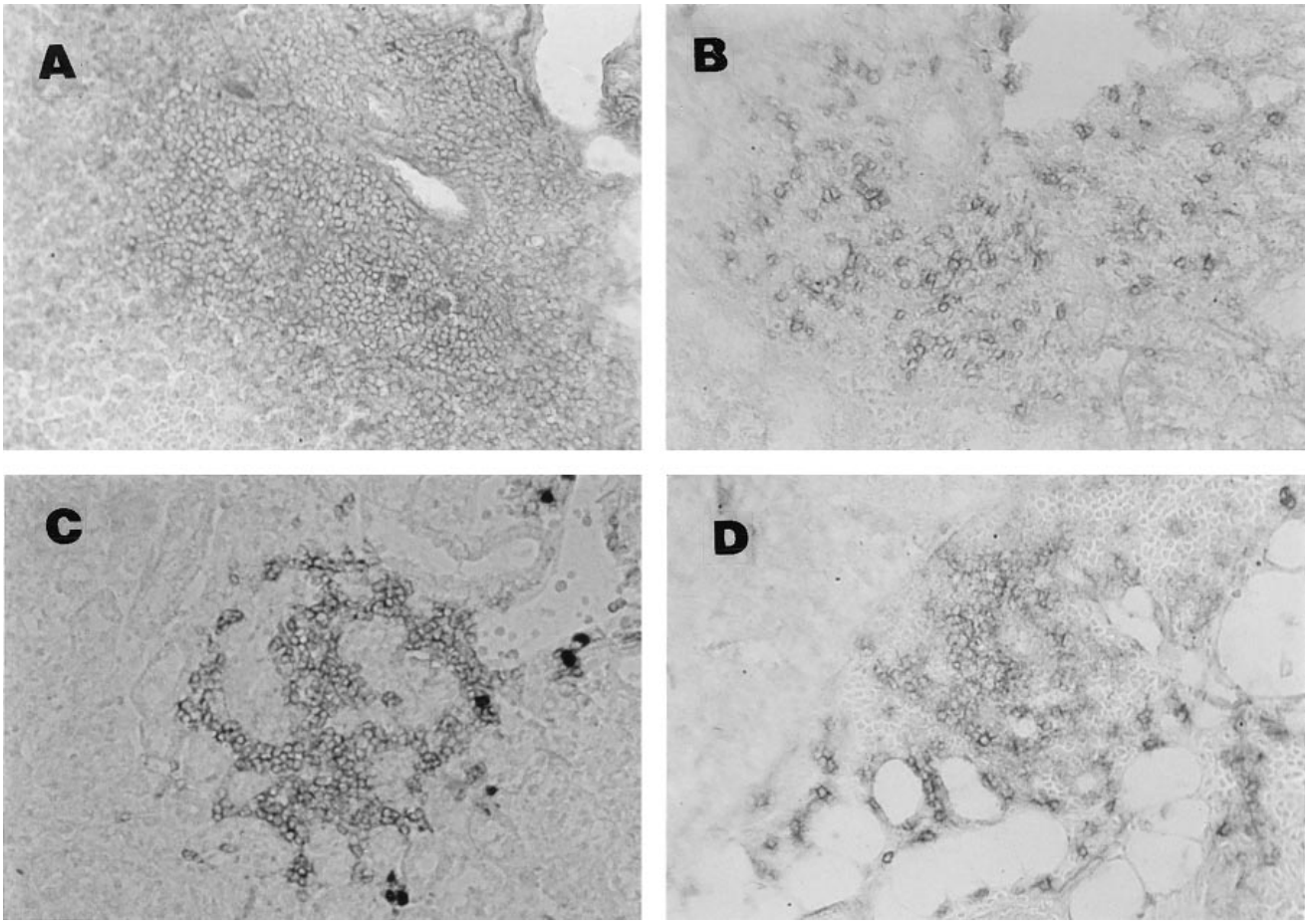

Fig. 3. Immunohistochemical detection of infiltrating mononuclear cells in the lacrimal glands from 14 weeks old. A large proportion of cells was positive for CD4 (A), and a variable number of cells was positive for IL-2 (B), IFN-γ (C), and IL-10 (D). (A–D, Immunoperoxidase staining, $\times$240.)

Table 2. Kinetic analysis of cytokine gene expression in the lacrimal gland and spleen by reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis

		NOD mice (week)					
Organ	Cytokine	4	6	8	16	20	30
Lacrimal gland	IL-1β	+	++	++	+++	+++	+++
	TNF-α	+	+	+	+	+	++
	IL-2	−	+	+	++	++	++
	IFN-γ	−	+	++	++	++	++
	IL-4	−	−	+	+	+	++
	IL-6	−	+	+	+	+	++
	IL-10	+	++	++	++	+++	+++
	IL-12	+	++	+++	+++	+++	+++
Spleen	IL-1β	++	++	++	++	++	++
	TNF-α	+	+	+	++	++	++
	IL-2	+	++	++	++	++	++
	IFN-γ	+	+	++	++	++	++
	IL-4	+	+	+	++	++	++
	IL-6	+	++	++	++	++	++
	IL-10	++	++	++	++	++	++
	IL-12	+	+	++	++	++	++

+, ++, +++: amount of PCR product detectable.
TNF-α, Tumour necrosis factor-alpha.

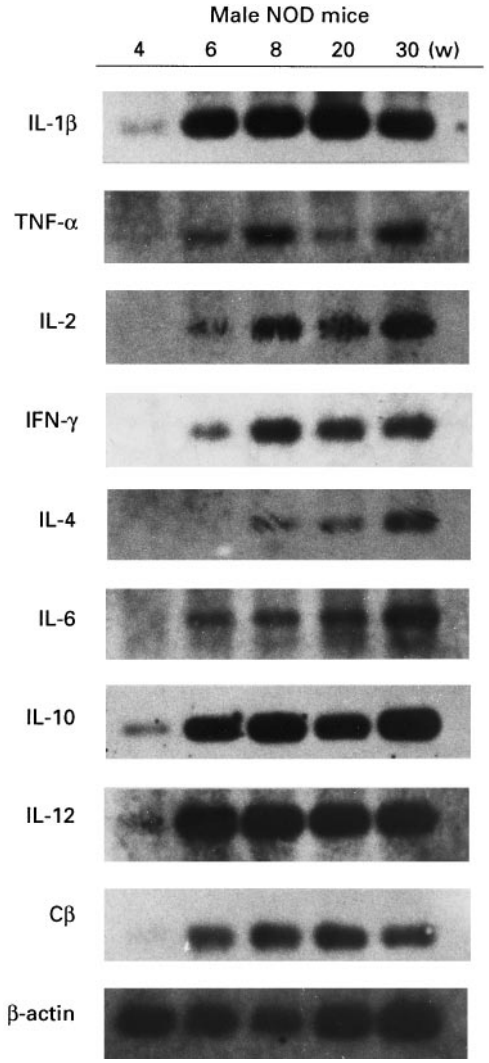

Fig. 4. Expression of cytokine, Cβ, and β-actin mRNA in the lacrimal glands of male NOD mice of various ages demonstrated by Southern blot analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products. TNF-α, Tumour necrosis factor-alpha.

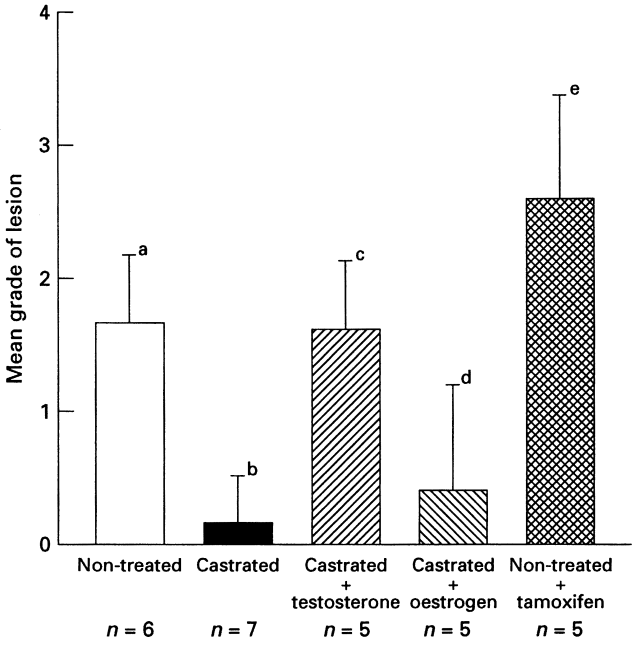

Fig. 5. Effect of castration and administration of sex steroids in male NOD mice. Castration was performed at 4 weeks old, and mice with or without administration of sex steroids were examined histopathologically at 8 weeks old. Mean grade of lesions was expressed as described according to the method of White & Casarett [26]. Statistically significant at $P < 0{\cdot}01$ between a and b, b and c, b and e, d and e (Student's *t*-test).

Expression of cytokine genes

In the lacrimal glands of male NOD mice at 4 weeks old before the onset of inflammatory lesions, we detected significant levels of cytokine mRNA expression of IL-1β, TNF-α, IL-10, and IL-12 p40. At 6 weeks old, when mild inflammatory lesions develop in the lacrimal glands, mRNA expression of IL-2, IL-6 and IFN-γ was also observed. The expression of these cytokine genes was consistently detected in the lacrimal glands during the course of disease until 30 weeks old, but IL-4 mRNA expression was detected later at 8 weeks old or more. Figure 4 and Table 2 show the expression of cytokine genes at various ages determined by RT-PCR and Southern blot analysis. No significant expression of these cytokine genes examined was detected in the lacrimal glands of female NOD mice at any age.

Effect of castration and administration of sex steroids

Figure 5 summarizes the effect of castration and administration of sex steroid in male NOD mice. Castrated male NOD mice at 4 weeks old displayed a significant decrease in incidence of autoimmune dacryoadenitis observed at 8 weeks old ($P < 0{\cdot}01$). Castrated NOD mice treated with testosterone showed increased incidence of autoimmune lesions in the lacrimal glands ($P < 0{\cdot}01$). In addition, oestrogen treatment with castration did not increase the incidence, but tamoxifen treatment without castration significantly increased the incidence of autoimmune dacryoadenitis ($P < 0{\cdot}01$). The castration and testosterone/tamoxifen manipulation did not affect the development of insulitis and the worsening of the sialadenitis in male NOD mice.

DISCUSSION

The present study demonstrates that a high incidence of autoimmune lesions in the lacrimal glands was observed exclusively in male NOD mice at any age. Autoimmune dacryoadenitis in male NOD mice is assumed to be histologically similar to that occurring in various animals models of SS [19–24]. SS is an autoimmune disorder with an unknown etiology, clinically characterized by progressive dryness such as keratoconjunctivitis sicca (dry eye), and xerostomia (dry mouth), termed 'sicca syndrome' [25,28]. Hypofunction in the lacrimal and salivary glands is due to lymphocytic infiltration, in which it is generally assumed that autoreactive T cells may recognize unknown self antigen and play a central role in the disease pathogenesis of SS [29]. In various animals models of SS, female preponderance has been shown in both dacryoadenitis and sialadenitis [17,18,22,23]. In autoimmune sialadenitis of NOD mice, we clearly demonstrated an increased incidence in females over males during the course of autoimmune disease [30]. On the other hand, we found a male preponderance of autoimmune lesions exclusively in the lacrimal glands of NOD mice.

Experimental autoimmune diseases appear to be influenced by sex steroids in a non-uniform manner. In spontaneous autoimmune murine lupus, female mice display higher autoantibody titres, as well as more severe disease manifestations, than do male mice [31]. In NZB/NZW mice, the strain in which sex-mediated mechanisms have been most extensively investigated, these effects are largely due to the actions of oestrogenic hormones, whereas testosterone suppresses both lupus development and autoantibody production [32]. There are experimental autoimmune conditions in which female sex hormones suppress, rather than enhance, disease developments such as adjuvant arthritis, experimental thyroiditis, and experimental autoimmune encephalomyelitis (EAE) [33–35]. In contrast, it is also known that DBA/1 male mice display a higher incidence of arthritis than do females after immunization with heterologous type II collagen [36,37], and arthritis develops exclusively in males after immunization with homologous type II collagen [38]. An increased frequency of collagen-induced arthritis (CIA) in males *versus* females was suggested, in that male mice in general are more susceptible than females to CIA. Since the total arthritis incidence and the relative difference in arthritis incidence between males and females differed between the MHC-identical strains analysed, CIA in mice is under the influence of genes that are associated neither with MHC nor with sex chromosomes. A similar preponderance for autoimmune disease in mice has been reported for the lupus-prone BXSB strain [31]. In the BXSB mouse, the male preponderance for disease has been shown to be caused by genes, present on the Y chromosome, that exert accelerating effects on lupus development [39]. Since castration did not change the frequency of autoimmune arthritis in males, it appears that male sex hormones exert a very small effect on arthritis in DBA/1 mice [40]. In the present study, castration in male NOD mice significantly decreased the incidence of autoimmune dacryoadenitis, and testosterone treatment with castration also increased the incidence of autoimmune lesions. Oestrogen treatment with castration did not increase the incidence, but tamoxifen treatment without castration significantly increased the incidence of autoimmune dacryoadenitis. These results indicate that the development and progression of autoimmune dacryoadenitis in male NOD mice is closely dependent on sex steroids, particularly testosterones. However, it remains unclear whether the opposite incidence of autoimmune lesions in the lacrimal and salivary glands is observed in the same strain. We can speculate that testosterone action appears to be organ-specific and to be mediated through local microenvironmental processes in the lacrimal gland of NOD mice. It was also demonstrated experimentally that testosterones may act through tissue-specific processes to suppress lacrimal gland autoimmunity [20]. Further studies on the mechanisms responsible for suppression of lacrimal gland autoimmune disease in females focused on the effects mediated by the gonads.

Although many previous studies of autoimmune animal models have been attempted in order to investigate the role of cytokines [41–43], those in autoimmune dacryoadenitis of the NOD mouse have not been analysed. With a RT-PCR and Southern blot method, we detected significant cytokine mRNA levels for IL-1β, TNF-α, IL-2, IFN-γ, IL-10 and IL-12 p40 in the lacrimal glands of male NOD mice from an early stage of the disease. Increases in gene expression of inflammatory cytokines (IL-1β, TNF-α, IL-6) have been demonstrated in various organ-specific autoimmune diseases [44–46]. It is now generally accepted that $CD4^+$ Th1 cells are important for the initiation of murine organ-specific autoimmune diseases [47,48]. NOD T cells in pancreas produce large amount of IFN-γ in response to this protein, and anti-IFN-γ antibodies can prevent the development of diabetes by adoptive transfer of diabetogenic cells [49]. Th1-type $CD4^+$ T cells appear to be involved in both early and late phases of diabetes development in the NOD mouse [50]. The transcription of IFN-γ in the lacrimal glands of male NOD mice probably plays a key role in the induction of class II molecule. IL-10 mRNA expression was associated with detectable IL-2 and IFN-γ mRNA expression. This pattern of cytokine mRNA expression in autoimmune dacryoadenitis differs from that reported in organ-specific autoimmune diseases such as EAE and diabetes in NOD mice. We have recently demonstrated that IL-10 and IL-12 play a major role on the initial phase of development of autoimmune sialadenitis in MRL/lpr mice [51]. We detected local expression of IL-10 and IL-12 p40 mRNA throughout the autoimmune dacryoadenitis in male NOD mice. Although IL-10 is known to have multiple suppressive effects on various effector phases of the immune response, recent reports have demonstrated that IL-10 accelerates *in vivo* the development of autoimmune lesions in NOD insulitis and NZB/WF_1 mice [52,53]. It was also reported that Th1 development of naive $CD4^+$ T cells depends on the coordinate action on IL-12 and IFN-γ [54]. Since IL-12 p40 has a striking effect on increasing production of IFN-γ from T cells [55], local IL-12 production may play an important role in the development of Th1-type autoimmune dacryoadenitis through IFN-γ production.

In conclusion, we have demonstrated that the high incidence of spontaneously occurring autoimmune dacryoadenitis in male NOD mice is dependent on sex steroids, and that local up-regulation of cytokines, including IL-10 and IL-12, may be involved in the cascade of events that initiate and accelerate Th1-type organ-specific autoimmunity in the lacrimal glands of male NOD mice.

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