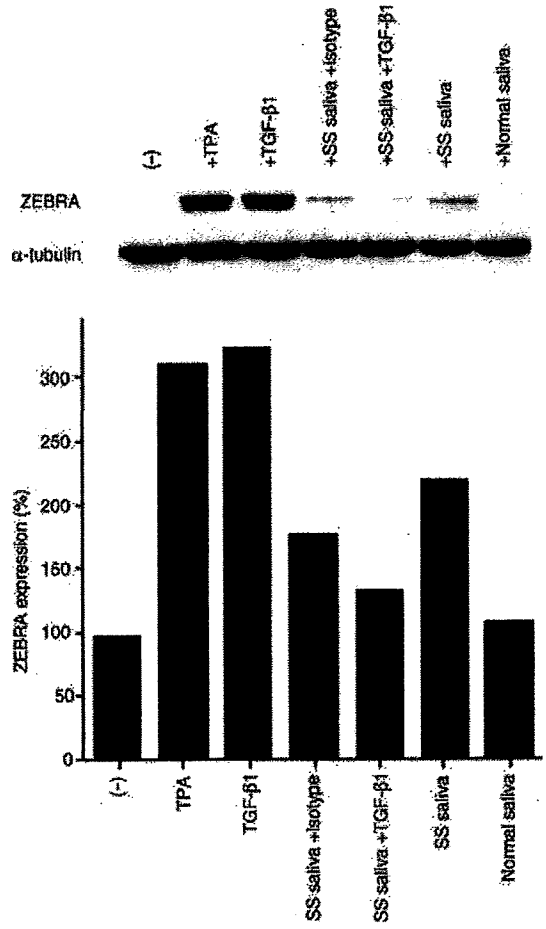

Figure 5. Effect of Sjogren's syndrome (SS) saliva on the expression of ZEBRA in Epstein–Barr virus (EBV)-positive B95-8 cells. B95-8 cells were treated for 48 hr with 25 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or 5 ng/ml of transforming growth factor-β1 (TGF-β1), or cells were cultured with SS saliva and a normal control for 48 hr. The neutralizing anti-TGF-β1 (10 μg/ml) was added at the start of the culture. ZEBRA bands were quantified by densitometric analysis. The results were calculated and the amount of ZEBRA was expressed as described in the Materials and methods. Tubulin was used for normalizing the amount of protein loaded. The results shown are representative of three independent experiments.

is not the sole activation factor, but a major factor of ZEBRA expression by SS saliva because TGF-β1-specific antibody inhibited the expression of ZEBRA in SS saliva, as shown by Western blotting.

Furthermore, to investigate the effect of SS saliva on expression of ZEBRA protein, we used EBV-positive B95-8 cells in which ZEBRA protein can be induced under several conditions (i.e. by TPA and TGF-β1). B95-8 cells were exposed to SS saliva, which had a high TGF-β1 concentration of 920 pg/ml. As shown in Fig. 5, ZEBRA protein was detected following treatment with SS saliva, suggesting that SS saliva contains factors that induce EBV reactivation. Neutralizing anti-TGF-β1 inhibited the induction of ZEBRA by SS saliva, in contrast to isotype-matched control antibody (Fig. 5). These results suggest that TGF-β1 may play a role in the EBV reactivation of a subpopulation in SS.

DISCUSSION

In the pathogenesis of SS, a role for EBV reactivation has been suggested.[1–4,6] EBV is a ubiquitous human herpesvirus that replicates in the salivary glands during primary infection[34] and the virus remains latent at this site in normal adults.[35] Initiation of the EBV lytic cycle is dependent on transcription of the BZLF1 gene. Although the viral lytic cycle can be induced by various reagents *in vitro*, including TPA,[16] calcium ionophore,[17] anti-IgG,[18] n-butyrate[19] and NO inhibitor,[22] it remains unclear how EBV reactivation is induced in SS. In this study, in order to clarify the association of EBV reactivation in SS pathogenesis and further analyse the mechanism of EBV reactivation, we modelled the reactivation of EBV, which latently infected the salivary gland, using plasmid DNA containing the BZLF1 promoter upstream from the luciferase reporter gene.

The Zp-luc(−221) reporter system has been widely used as a marker of EBV reactivation, even in EBV-negative cells.[36] However, it is well known that the induction of promoter activity by TPA, or TGF-β1, in EBV-negative cells is relatively low.[33,36] These results might be largely a result of the absence of ZEBRA in these assay systems. Regulation of ZEBRA is known to be achieved by two steps.[27] Initial activation of the BZLF1 promoter is weak, but this level of ZEBRA sufficiently induces full activation of the promoter by means of self-promoter bindings. Therefore, the weak fold-induction of Zp-luc seen in our system might correspond to the initial 'weak' activations prior to the occurrence of full activation.

We demonstrated the EBV reactivation model in SS and found that expression of the BZLF1 gene in glandular epithelial cells might be induced via MAPK and calcium/calmodulin dependent-kinase pathways, as previously demonstrated in B lymphocytes.[29,30] However, the physiological stimuli responsible for the EBV activation in SS have not yet been characterized.

Our results showed that treatment with saliva from SS patients increases the reactivation ability of EBV as compared with normal saliva samples. This suggests the existence of factors that are able to induce the reactivation of EBV. It has been reported that infectious EBV is present in the saliva of SS patients[1] and in HHV6 superinfection *in vitro*,[23] but we considered that these viruses were not present in the saliva samples used in the present study, as a result of the saliva processing technique used (centrifugation for 45 min at 12 000 *g* to remove cells and particulate debris, followed by filtering through a 0·22-μm filter). We thus assumed that some soluble factors play a major role in the reactivation of EBV.

The expression of ZEBRA can be accomplished, *in vitro*, by treatment of latent EBV-positive B cells with various activating agents, including TGF-β1, TPA, butyrate, calcium ionophores and anti-IgG. These treatments trigger a variety of cellular signalling pathways, resulting in the activation of cellular transcription factors stimulating transcription from the BZLF1 promoter, Zp.[27,30,37,38] Zp can be activated through PKC and calcium/calmodulin-dependent protein kinase directly cross-linking via anti-IgG.[39] Anti-IgG also induced rapid phosphorylation of MAPK in Akata cells.[30] Moreover, MAPK was

involved in the activation of BZLF1 induced by TGF-β1 in Raji and B95-8 cells.[33] In P3HR-1 and Rael cells, however, MAPK was not involved in the activation of Zp-luc by TGF-β1.[40] This discrepancy might be explained by different characteristics of cell lineage. The signal transduction of Zp-luc activation in salivary gland cells has not been reported. We investigated the SS saliva signal in EBV reactivation in our models by using specific inhibitors of intracellular signals. A specific inhibitor of PKC did not affect the SS saliva-induced Zp-luc activity, whereas treatment with inhibitors of calmodulin, calcineurin, IP_3 and MAPK, dose dependently decreased this induction. This implies that the effect of SS saliva on BZLF1 expression requires calcium/calmodulin and/or the MAPK pathway.

TGF-β1 has been shown to exert its effects through a wide range of intracellular routes. Recent studies from several laboratories have reported that Smads are intermediate effector proteins which transduce the TGF-β1 signal from the plasma membrane to the nucleus.[41] TGF-β1 can induce gene expression via c-Jun N-terminal kinase (JNK) activation[42,43] or p38 MAPK.[44] The role of MAPK/ERK in the TGF-β1 signalling pathway has been described.[45] PKC has also been shown to be involved in TPA- and anti-IgG-induced EBV reactivation[30,46] and could play an important role in the signal transduction by TGF-β1.[47,48] This suggests that SS saliva might contain some soluble factor(s), such as TGF-β1, which can induce virus reactivation.

It is known that, in contrast to normal salivary glands, SS salivary glands express increased levels of cytokines.[31,49] Furthermore, recent reports have suggested that in latently infected B cells, the lytic cycle can be induced by TGF-β1[24] and that salivary TGF-β1 concentrations are elevated in SS.[31] In our experiments, TGF-β1 increased Zp-luc activity, and TGF-β1 concentrations were significantly elevated in SS saliva as compared to saliva from healthy volunteers (Fig. 4). Furthermore, we investigated the effect of SS saliva on Zp activation in B cells by Western blot analysis, using an SS saliva sample with a high concentration of TGF-β1. ZEBRA was detected upon exposure to SS saliva (Fig. 5), suggesting that SS saliva contains factors which induce viral reactivation. As shown in Fig. 5, neutralizing anti-TGF-β1 inhibited ZEBRA induction by SS saliva. In SS saliva, TGF-β1 may play a major role in viral reactivation.

In conclusion, we have shown that SS saliva induces Zp-luc activity and that calcium/calmodulin and/or the MAPK pathway is required for this induction, as demonstrated through the use of specific signalling inhibitors. These findings may be of importance for developing new strategies to inhibit EBV reactivation in SS.

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REFERENCES

1 Saito I, Servenius B, Compton T, Fox RI. Detection of Epstein–Barr virus DNA by polymerase chain reaction in blood and tissue biopsies from patients with Sjogren's syndrome. J Exp Med 1989; **169:** 2191–8.

2 Mariette X, Gozlan J, Clerc D, Bisson M, Morinet F. Detection of Epstein–Barr virus DNA by *in situ* hybridization and polymerase chain reaction in salivary gland biopsy specimens from patients with Sjogren's syndrome. Am J Med 1991; **90:**286–94.

3 Saito I, Nishimura S, Kudo I, Fox RI, Moro I. Detection of Epstein–Barr virus and human herpes virus type 6 in saliva from patients with lymphoproliferative diseases by the polymerase chain reaction. Arch Oral Biol 1991; **36:**779–84.

4 Fox RI, Pearson G, Vaughan JH. Detection of Epstein–Barr virus-associated antigens and DNA in salivary gland biopsies from patients with Sjogren's syndrome. J Immunol 1986; **137:**3162–8.

5 Fox RI, Bumol T, Fantozzi R, Bone R, Schreiber R. Expression of histocompatibility antigen HLA-DR by salivary gland epithelial cells in Sjogren's syndrome. Arthritis Rheum 1986; **29:**1105–11.

6 Tateishi M, Saito I, Yamamoto K, Miyasaka N. Spontaneous production of Epstein–Barr virus by B lymphoblastoid cell lines obtained from patients with Sjogren's syndrome. Possible involvement of a novel strain of Epstein–Barr virus in disease pathogenesis. Arthritis Rheum 1993; **36:**827–35.

7 Sullivan JL. Epstein–Barr virus and lymphoproliferative disorders. Semin Hematol 1988; **25:**269–79.

8 Inoue N, Harada S, Miyasaka N, Oya A, Yanagi K. Analysis of antibody titers to Epstein–Barr virus nuclear antigens in sera of patients with Sjogren's syndrome and with rheumatoid arthritis. J Infect Dis 1991; **164:**22–8.

9 Yamaoka K, Miyasaka N, Yamamoto K. Possible involvement of Epstein–Barr virus in polyclonal B cell activation in Sjogren's syndrome. Arthritis Rheum 1988; **31:**1014–21.

10 Inoue H, Tsubota K, Ono M *et al.* Possible involvement of EBV-mediated alpha-fodrin cleavage for organ-specific autoantigen in Sjogren's syndrome. J Immunol 2001; **166:**5801–9.

11 Haneji N, Nakamura T, Takio K *et al.* Identification of alpha-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science 1997; **276:**604–7.

12 Rickinson AB, Moss DJ, Wallace LE *et al.* Long-term T-cell-mediated immunity to Epstein–Barr virus. Cancer Res 1981; **41:**4216–21.

13 Slaughter L, Carson DA, Jensen FC, Holbrook TL, Vaughan JH. *In vitro* effects of Epstein–Barr virus on peripheral blood mononuclear cells from patients with rheumatoid arthritis and normal subjects. J Exp Med 1978; **148:**1429–34.

14 Countryman J, Miller G. Activation of expression of latent Epstein–Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc Natl Acad Sci USA 1985; **82:**4085–9.

15 Rooney CM, Rowe DT, Ragot T, Farrell PJ. The spliced BZLF1 gene of Epstein–Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J Virol 1989; **63:**3109–16.

16 zur Hausen H, O'Neill FJ, Freese UK, Hecker E. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. Nature 1978; **272:**373–5.

17 Faggioni A, Zompetta C, Grimaldi S, Barile G, Frati L, Lazdins J. Calcium modulation activates Epstein–Barr virus genome in latently infected cells. Science 1986; **232:**1554–6.

18 Takada K. Cross-linking of cell surface immunoglobulins induces Epstein–Barr virus in Burkitt lymphoma lines. Int J Cancer 1984; **33:**27–32.

19 Luka J, Kallin B, Klein G. Induction of the Epstein–Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology 1979; **94:**228–31.

20 Meuller-Lantzsch N, Georg B, Yamamoto N, zur Hausen H. Epstein–Barr virus-induced proteins. II. Analysis of surface polypeptides from EBV-producing and -superinfected cells by immunoprecipitation. Virology 1980; **102:**401–11.

21 Mueller-Lantzsch N, Georg B, Yamamoto N, zur Hausen H. Epstein–Barr virus-induced proteins. III. Analysis of polypeptides from P3HR-1-EBV-superinfected NC37 cells by immunoprecipitation. Virology 1980; **102:**231–3.
22 Mannick JB, Asano K, Izumi K, Kieff E, Stamler JS. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein–Barr virus reactivation. Cell 1994; **79:**1137–46.
23 Flamand L, Stefanescu I, Ablashi DV, Menezes J. Activation of the Epstein–Barr virus replicative cycle by human herpesvirus 6. J Virol 1993; **67:**6768–77.
24 di Renzo L, Altiok A, Klein G, Klein E. Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. Int J Cancer 1994; **57:**914–9.
25 Fox RI, Saito I. Criteria for diagnosis of Sjogren's syndrome. Rheum Dis Clin North Am 1994; **20:**391–407.
26 Yanagawa T, Hayashi Y, Nagamine S, Yoshida H, Yura Y, Sato M. Generation of cells with phenotypes of both intercalated duct-type and myoepithelial cells in human parotid gland adenocarcinoma clonal cells grown in athymic nude mice. Virchows Arch B Cell Pathol Incl Mol Pathol 1986; **51:**187–95.
27 Flemington E, Speck SH. Autoregulation of Epstein–Barr virus putative lytic switch gene BZLF1. J Virol 1990; **64:**1227–32.
28 Flemington E, Speck SH. Identification of phorbol ester response elements in the promoter of Epstein–Barr virus putative lytic switch gene BZLF1. J Virol 1990; **64:**1217–26.
29 Mellinghoff I, Daibata M, Humphreys RE, Mulder C, Takada K, Sairenji T. Early events in Epstein–Barr virus genome expression after activation. regulation by second messengers of B cell activation. Virology 1991; **185:**922–8.
30 Daibata M, Speck SH, Mulder C, Sairenji T. Regulation of the BZLF1 promoter of Epstein–Barr virus by second messengers in anti-immunoglobulin-treated B cells. Virology 1994; **198:**446–54.
31 Fox PC, Grisius MM, Bermudez DK, Sun D. Cytokine mRNA expression in labial salivary glands and cytokine secretion in parotid saliva in Sjogren's syndrome. Adv Exp Med Biol 1998; **438:**909–15.
32 Fox PC, Brennan M, Di Sun P. Cytokine expression in human labial minor salivary gland epithelial cells in health and disease. Arch Oral Biol 1999; **44** (Suppl. 1):S49–52.
33 Fahmi H, Cochet C, Hmama Z, Opolon P, Joab I. Transforming growth factor beta 1 stimulates expression of the Epstein–Barr virus BZLF1 immediate-early gene product ZEBRA by an indirect mechanism which requires the MAPK kinase pathway. J Virol 2000; **74:**5810–8.
34 Morgan DG, Niederman JC, Miller G, Smith HW, Dowaliby JM. Site of Epstein–Barr virus replication in the oropharynx. Lancet 1979; **2:**1154–7.
35 Chang RS, Lewis JP, Abildgaard CF. Prevalence of oropharyngeal excreters of leukocyte-transforming agents among a human population. N Engl J Med 1973; **289:**1325–9.
36 Niller HH, Salamon D, Uhlig J *et al.* Nucleoprotein structure of immediate-early promoters Zp and Rp and of oriLyt of latent Epstein–Barr virus genomes. J Virol 2002; **76:**4113–8.
37 Farrell PJ, Rowe DT, Rooney CM, Kouzarides T. Epstein–Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J 1989; **8:**127–32.
38 Shimizu N, Takada K. Analysis of the BZLF1 promoter of Epstein–Barr virus: identification of an anti-immunoglobulin response sequence. J Virol 1993; **67:**3240–5.
39 Daibata M, Mellinghoff I, Takagi S, Humphreys RE, Sairenji T. Effect of genistein, a tyrosine kinase inhibitor, on latent EBV activation induced by cross-linkage of membrane IgG in Akata B cells. J Immunol 1991; **147:**292–7.
40 Liang CL, Chen JL, Hsu YP, Ou JT, Chang YS. Epstein–Barr virus BZLF1 gene is activated by transforming growth factor-beta through cooperativity of Smads and c-Jun/c-Fos proteins. J Biol Chem 2002; **277:**23345–57.
41 Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1997; **390:**465–71.
42 Hocevar BA, Brown TL, Howe PH. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J 1999; **18:**1345–56.
43 Wang W, Zhou G, Hu MC, Yao Z, Tan TH. Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor beta (TGF-beta)-activated kinase (TAK1), a kinase mediator of TGF beta signal transduction. J Biol Chem 1997; **272:**22771–5.
44 Hannigan M, Zhan L, Ai Y, Huang CK. The role of p38 MAP kinase in TGF-beta1-induced signal transduction in human neutrophils. Biochem Biophys Res Commun 1998; **246:**55–8.
45 Yamaguchi K, Shirakabe K, Shibuya H *et al.* Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science 1995; **270:**2008–11.
46 Davies AH, Grand RJ, Evans FJ, Rickinson AB. Induction of Epstein–Barr virus lytic cycle by tumor-promoting and non-tumor-promoting phorbol esters requires active protein kinase C. J Virol 1991; **65:**6838–44.
47 Halstead J, Kemp K, Ignotz RA. Evidence for involvement of phosphatidylcholine-phospholipase C and protein kinase C in transforming growth factor-beta signaling. J Biol Chem 1995; **270:**13600–3.
48 Suzuki M, Asplund T, Yamashita H, Heldin CH, Heldin P. Stimulation of hyaluronan biosynthesis by platelet-derived growth factor-BB and transforming growth factor-beta 1 involves activation of protein kinase C. Biochem J 1995; **307:**817–21.
49 Fox RI, Kang HI, Ando D, Abrams J, Pisa E. Cytokine mRNA expression in salivary gland biopsies of Sjogren's syndrome. J Immunol 1994; **152:**5532–9.

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Development of Autoimmune Exocrinopathy Resembling Sjögren's Syndrome in Estrogen-Deficient Mice of Healthy Background

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Although a number of autoimmune diseases are known to develop in postmenopausal women, the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear. We speculate that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens. Previously, we have identified 120-kd α-fodrin as an important autoantigen in Sjögren's syndrome (SS). When healthy C57BL/6 (B6) mice were treated with an ovariectomy (Ovx), we found a significant increase in TUNEL$^+$-apoptotic epithelial cells in the salivary gland cells associated with α-fodrin cleavage during 2 and 3 weeks after Ovx. By contrast, no apoptotic cells were found in estrogen receptor-α knockout mice. In *in vitro* studies using primary cultured mouse salivary gland cells and human salivary gland cells, we found a cleavage product of 120-kd α-fodrin in cells that had undergone tamoxifen (Tam)-induced apoptosis through caspase activation, especially caspase-1. Adoptive transfer of α-fodrin-reactive T cells into Ovx-B6 and -SCID mice resulted in the development of autoimmune exocrinopathy quite similar to SS. These results suggest that estrogen deficiency exerts a crucial influence on autoantigen cleavage, and may cause, in part, autoimmune exocrinopathy in postmenopausal women. *(Am J Pathol 2003, 163:1481–1490)*

Loss of ovarian function following menopause results in functional failures of the immune system, bone metabolism, and endocrine system. Estrogenic action has been suggested to be responsible for the strong female preponderance of many autoimmune diseases, including systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), and Sjögren's syndrome (SS).[1–4] Sex hormones influence both humoral and cell-mediated immune responses in a number of experimental models.[5–9] Previous reports indicate that the increase in autoantibody production as a result of estrogen deficiency is mediated by cytokines such as interleukin-6 (IL-6), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), and that estrogen plays an important role in the regulation of B-lymphocyte development in mouse bone marrow.[10–12]

Recently, we have demonstrated that the dysfunction of regulatory T cells as a result of estrogen deficiency may play a crucial role on acceleration of organ-specific autoimmune lesions, and that estrogenic action influences target epithelial cells through Fas-mediated apoptosis in a murine model for SS.[13] Although autoimmune diseases are triggered by various environmental factors, such as hormonal changes, microbial infections, stress, and aging,[14,15] much less is known about the role of estrogen deficiency on the formation of autoantigen. We hypothesize that estrogen deficiency may influence the formation of pathogenic autoantigen in target organs through a T-cell-independent pathway.

Previously, we have identified 120-kd α-fodrin as an important autoantigen in both NFS/*sld* murine SS model and in patients,[16] but the mechanisms of α-fodrin cleavage in the salivary gland cells remain unclear. Our recent study has been strongly suggestive of essential roles of caspase cascade for α-fodrin cleavage leading to tissue destruction in primary SS.[17] α-fodrin is a ubiquitous, heterodimeric calmodulin-binding protein that is cleaved by calcium-activated protease (calpain) in apoptotic cells and caspase through Fas-mediated apoptosis in Jurkat cells.[18–20] The fodrin α-subunit of various cells has also been shown to be cleaved in association with apoptosis.[21–23] Several reports have demonstrated that estrogen may play an inhibitory role on apoptosis in endothelial cells, breast cancer cells, cardiac myocytes, prostate cells, and neuronal cells.[24–27] Moreover, it has been noted that some enzymatic activities are elevated in postmenopausal women compared with normal healthy women.[28,29]

The aim of this study was to analyze the effect of estrogen deficiency on the formation of pathogenic autoantigen. Moreover, caspase activity in mouse salivary

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gland cells stimulated by an antiestrogenic action has been analyzed, indicating that estrogen deficiencies may play a pivotal role in autoantigen cleavage initially triggered in the salivary and lacrimal gland.

Materials and Methods

Mice and Treatments

Female C57BL/6 (B6) mice (H-2^b) were purchased from Japan SLC (Shizuoka, Japan), and maintained in a specific pathogen-free (SPF) mouse colony and given food and water *ad libitum*. Estrogen receptor-α knockout mice (ERαKO) on a B6 strain background were purchased from Taconic (Germantown, NY). Female SCID C.B-17-scid/scid mice (H-2^d), purchased from Japan SLC (Shizuoka), were used to confirm cell transfer experiment. Normal female B6 (H-2^b), and BALB/c mice (H-2^d), purchased from Japan SLC (Shizuoka), were used to obtain antigen-stimulated T cells. Mice were ovariectomized (Ovx) at 4 weeks of age and compared with sham-operated (Sham, in both strain) mice. At 1 to 6 weeks after Ovx, all organs were removed from the mice and analyzed.

Histological Analysis

All organs were removed from mice, fixed with 4.0% phosphate-buffered formaldehyde (pH 7.2), and prepared for histological examination. The sections were stained with hematoxylin and eosin. The disease incidence was determined using the histological score of inflammatory lesions by White and Casarret,[30] estimated by three independent, well-trained pathologist in a blinded manner.

In Situ *End-Labeling of Fragmented DNA (TUNEL)*

Apoptotic cells were detected in sections using the *in situ* TUNEL Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Briefly, sections were incubated with proteinase K (20 μg/ml) for 10 minutes, and then presoaked in TdT buffer (0.5 μmol/L cacodylate, 1 μmol/L CoCl, 0.5 μmol/L dithiothreitol, 0.05% bovine serum albumin, 0.15 mol/L NaCl) for 10 minutes. Sections were incubated for 2 hours at 37°C in 25 ml of TdT solution, containing 1X terminal transferase buffer, 0.5 nmol of biotin-dUTP, and 10 U of TdT (Wako Pure Chemical). After the TdT reaction, sections were soaked in TdT blocking buffer (300 nmol/L NaCl, 30 mmol/L tri-sodium citrate-2-hydrate), incubated with HRP-conjugated streptavidin for 30 minutes at room temperature, and developed for 10 minutes in phosphate-buffered citrate (pH 5.8) containing 0.6 mg/ml DAB. Nuclei were counterstained with hematoxylin.

Western Blot Analysis

Westrn blot analysis with anti-human α-fodrin (Affiniti, Mamhead, UK) was performed. Briefly, the cells were incubated in 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L NaCl, 0.5% Triton X-100, 5 mmol/L ethylenediaminetetraacetate (EDTA), and 3 mmol/L MgCl2 lysis buffer. After centrifugation for 20 minutes at 12,000 × *g* at 4°C, supernatant was extracted and used for sample. Ten micrograms of each sample per well was used for sodium dodecyl sulfide-polyacrylamide gel electrophoresis. Protein binding was visualized with enhanced chemiluminescence Western blotting reagent (Amersham Biosciences, Arlington Heights, IL). Control for protein loading was provided by anti-human α-tubulin, or GAPDH monoclonal antibody (Sigma Chemical Co., St. Louis, MO). To detect caspase-1 in cultured human cells and mouse tissues, Western blot analysis was performed by the indicated methods using anti-human caspase-1 (Sigma Chemical Co.), and anti-human active form caspase-1 (p20 subunit; Upstate Biotech, Charlottesville, VA) polyclonal antibody. An anti-human caspase-1 polyclonal antibody is known to cross-react with mouse lysate.

Primary Culture of Mouse Salivary Gland (MSG) Cells

Mouse salivary gland (MSG) epithelial cells were prepared as previously described.[13,17] Briefly, mouse salivary glands were minced into 1-mm^2 pieces, washed with Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, and placed in a 60-mm dish containing HBSS with 0.76 μg/ml EDTA, 4.9 μg/ml L-ascorbic acid, and 4.9 μg/ml reduced glutathione. Fragments were washed with Dulbecco's modified Eagle's medium/soybean trypsin inhibitor, and placed in a mixture of collagenase (750 U/ml of type I) and hyarulonidase (500 U/ml of type IV) dissolved in DMEM/F12 containing 10% fetal bovine serum (FBS). The digest suspension was passed through a 100-μm nylon mesh filter. Adherant cells after culture in DMEM containing 10% FBS for 24 hours at 37°C were isolated as salivary gland epithelial cells. Apoptotic cells were detected by flow cytometry with an EPICS flow cytometer (Beckman Coulter, Miami, FL) using the Annexin V-FITC Apoptosis Detection kit (Genzyme, Cambridge, MA).

Cell Culture

MSG cells, human salivary gland adenocarcinoma cell line (HSG),[31] breast cancer cell line (MCF-7), colon cancer cells (HT29, Colo201), and Jurkat cells were cultured in the DMEM and RPMI-1640 including 10% FBS at 37°C. When the cells were treated with tamoxifen (Tam) (ICN Biochemicals, Costa Mesa, CA) and 17β-estradiol (Wako Pure Chemical), they were cultured in the medium without FBS. Apoptotic cells were detected by flow cytometry with an EPICS flow cytometer (Beckman Coulter) using the Annexin V-FITC Apoptosis Detection kit (Genzyme).

Cell Transfection

We used polymerase chain reaction (PCR) techniques to generate derivatives of a human caspase-1 promoter-

luciferase construct. The following forward and reverse oligomers were used as primers to create a promoter of the human caspase-1 gene: 5′CACTGCAGATTGAGAAACTCTTCACTG3′, 5′GATCTAGAGGCTTTTCTCTCCTCCCT3′. The PCR-amplified promoter fragments, including Pst-1/Xba-1 site, were cloned into the multiple cloning site of the pGL3-basic vector (Promega, Charbonnieres, France), upstream of the luciferase gene. The caspase-1 promoter-luciferase gene were transfected into HSG cells using LipofectAMIN (Promega). The vector pGL3-basic (lacking a promoter) and the vector (Promega) pGL3-control served as negative and positive controls, respectively. Briefly, the transfection medium, containing 10 μg of plasmid DNA and 60 μl of Lipofectin reagent in 2 ml of serum-free DMEM was incubated for 20 minutes at room temperature and then diluted with serum-free DMEM to a final volume of 5 ml and added to HSG cells, plated the day before. The transfection process occurred at 37°C for 5 hours, then 5 ml of DMEM containing 20% fetal calf serum (FCS) was added to the cells.

Luciferase Assay

The transfected cells were incubated for 24 hours and stimulated during the last 2 hours with Tam (1×10^{-7}M). Pretreatment with 17β-estradiol (1×10^{-9}M) was performed during last 12 hours. After rinsing with phosphate-buffered saline, cells were lysed with reporter lysis buffer (Promega), and cell extracts were used for luciferase assay with the Promega kit in a luminometer (Promega). To control transfection efficiency, pSVβ-galactosidase plasmid (Promega) was cotransfected with the luciferase reporter constructs in a 1:4 ratio. The results showed that the difference in the relative efficiency of transfection between constructs was negligible.

Caspase Activities

Caspase activities in Tam-induced apoptosis in HSG cell extracts and mouse various tissues were assayed using Caspase-Family Colorimetric Substrate Set (BioVision Inc., Palo Alto, CA). Briefly, tissue or cell lysates were incubated with pNA-conjugated substrates (200 mmol/L, caspase-1, -2, -3, -5, -6, -8, and -9 substrate: Ac-YVAD-pNA, Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-WEHD-pNA, Ac-VEID-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA) at 37°C for 2 hours. Absorbance of each sample was read at 405 nm in a microtiter plate reader using a 96-well plate. Fold-increase in caspase activity was determined by comparing these results with the level of the uninduced control. The caspase inhibitors z-VAD-fmk (1 μmol/L), Ac-DEVD-CHO (1 μmol/L), and Ac-WEHD-CHO (0.5 μmol/L) (Sigma Chemical Co.) were added to the Tam-stimulated HSG cells. After the incubation for 48 hours, apoptotic cells were detected using Annexin-V flow cytometric analysis.

Adoptive Transfer

To obtain α-fodrin-reactive T cells, B6 mice were injected subcutaneously with 20 μg recombinant α-fodrin protein (JS-1) and Freund's complete adjuvant (ICN Biochemicals) at 4 weeks of age, and i.p. injections of 20 μg JS-1 and Freund's incomplete adjuvant (ICN Biochemicals) were performed at 6 weeks of age. OVA (10 μg/head)-reactive T cells were obtained as the same manner for control experiments. After 2 weeks later (8 weeks of age), mice were sacrificed and the splenic T cells were obtained as donor cells. As recipients, female B6 mice were ovariectomized (Ovx, $n = 7$) or sham-operated (Sham, $n = 5$) at 4 weeks of age. After 2 weeks (6 weeks of age), Ovx- and Sham-mice were transferred i.p. with 5×10^6 α-fodrin-reactive T cells. The transferred mice were analyzed at 4 and 8 weeks after the cell transfer. To confirm cell transfer experiments, Ovx- and Sham-SCID mice ($n = 7$ and $n = 5$) were transferred with α-fodrin-reactive T cells obtained from BALB/c mice in the same manner.

Proliferation Assay

Splenic T cells were performed on proliferation assay against various antigens including recombinant α-fodrin protein (JS-1). Single cell suspensions were cultured in 96-well flat bottom microtiter plate (5×10^5 cells/well) in RPMI-1640 containing 10% FCS, penicillin/streptomycin, and β-mercaptoethanol. Cells were cultured with 10 μg/ml JS-1, and 2.0 μg/ml Con A (EY Laboratories, San Mateo, CA). The control antigens used were OVA (5 μg/ml), lysozyme (5 μg/ml), and albumin (5 μg/ml) (Sigma, St. Louis, MO).^{3}[H]thymidine incorporation during the last 20 hours of the culture was evaluated using an automatoid β-liquid scintillation counter.

Results

Apoptosis Induced in Salivary Gland Cells by Ovariectomy (Ovx)

To examine the *in vivo* effects of estrogen deficiency in normal B6 and ERαKO mice, Ovx was performed at the age of 4 weeks. A radioimmunoassay confirmed that β-estradiol was not detectable in the sera of Ovx-mice (Ovx-B6 and Ovx-ERαKO, not detected; Sham-B6, 27.4 ± 2.8 pg/ml; Sham-ERαKO, 26.8 ± 3.6 pg/ml). At 1 to 6 weeks after Ovx, an *in situ* apoptosis detection assay was performed using all organs. Although we found a significant increase in TUNEL$^+$ apoptotic cells in the salivary gland sections of B6 mice, but not ERαKO mice, at 2 or 3 weeks after Ovx (Figure 1), no significant apoptosis was observed in any other organs of Ovx and Sham mice. To define TUNEL labeling due to the effect of Ovx *in vivo*, we examined Annexin V-flow cytometric analysis using primary cultured MSG cells, indicating that a small proportion of apoptotic cells (12.5%) in Ovx mice was found, but not in Sham mice (3.1%) (Figure 1A). No significant difference in number of TUNEL$^+$ apoptotic cells of Ovx and Sham mice was observed at 0, 1, 4, 5, and 6 weeks after Ovx (Figure 1B). Thus, apoptotic changes in the salivary glands of normal mice were observed transiently at 2 or 3 weeks after Ovx, supposing

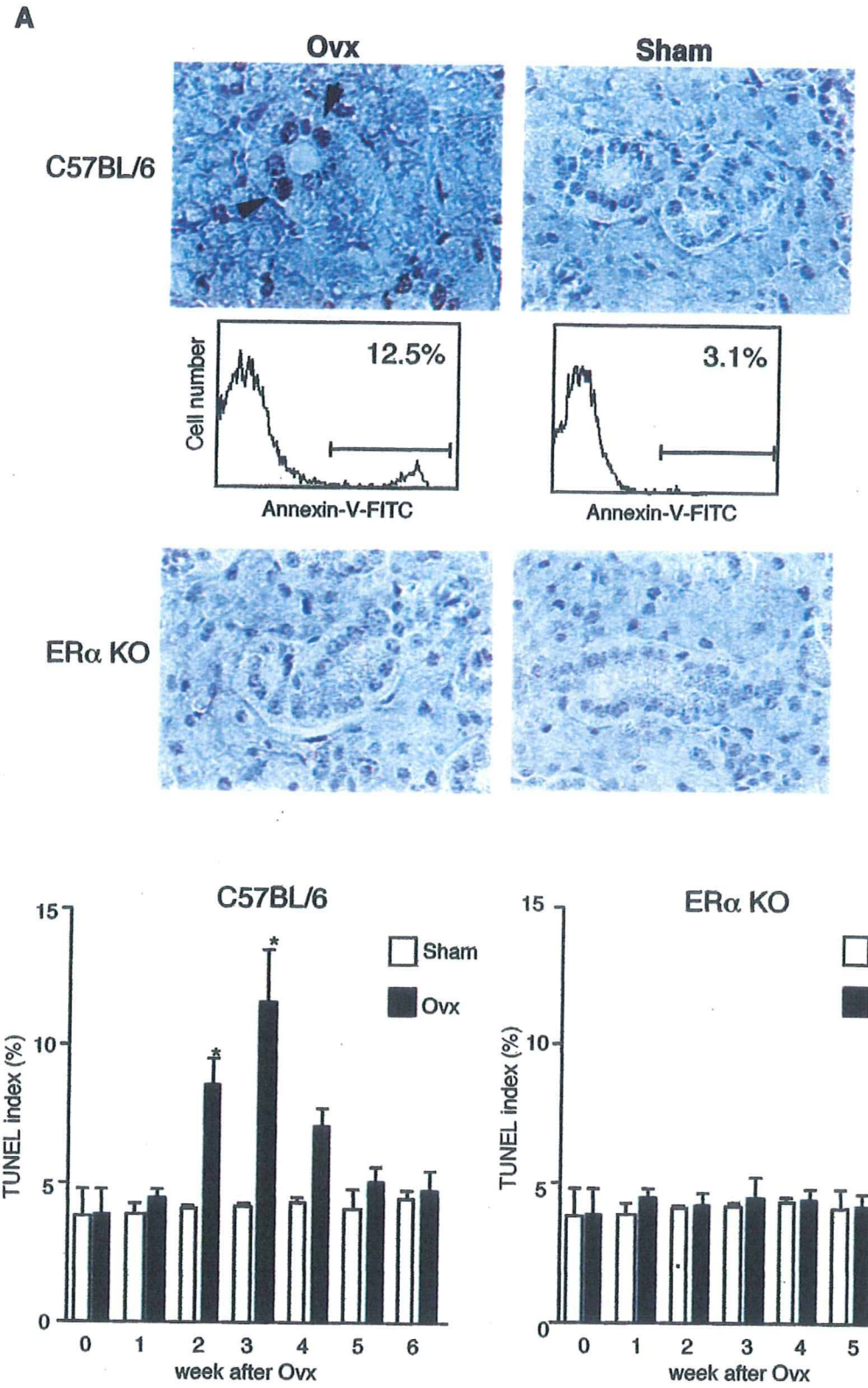


Figure 1. A: Detection of TUNEL$^+$-apoptotic cells in the salivary gland sections from Ovx- and Sham-B6 mice, but not from ERαKO mice, at 3 weeks after Ovx (**arrows**). Annexin V-flow cytometric analysis using MSG cells demonstrated 12.5% positive cells detected in Ovx-B6 mice, but 3.1% positive in Sham-B6 mice. Data are representative in triplicate. **B:** A significant increase of apoptotic epithelial cells was observed in the salivary gland tissues from Ovx-B6 mice, not from ERαKO mice, restricted at 2 and 3 weeks after Ovx. The percentage of epithelial cells staining positively with TUNEL was enumerated using a 10- × 20-grid net micrometer disk covering an objective of area 0.16 mm^2. Data were analyzed in 10 fields per section and expressed as mean percent ± SD in five mice examined per group. (*$P < 0.05$; Student's *t*-test.)

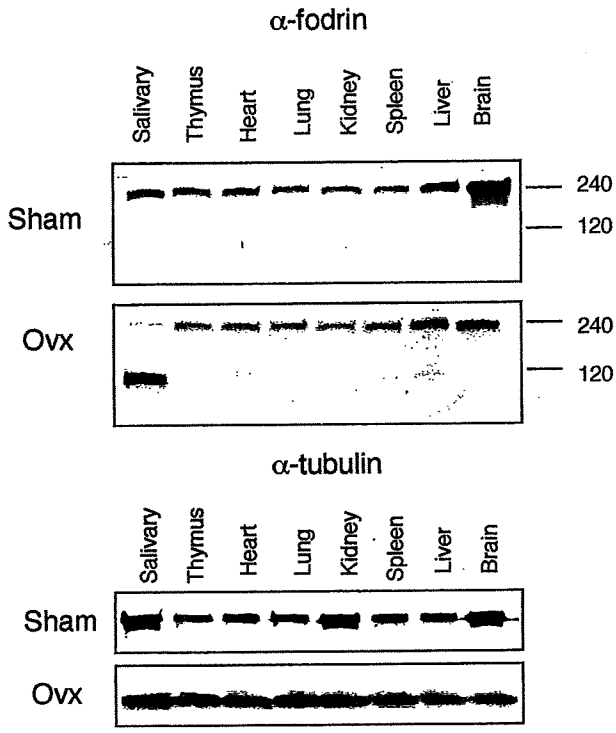


Figure 2. Detection of α-fodrin cleavage in various tissues from Ovx- and Sham-B6 mice on Western blot analysis showing distinct band of 120 kd in the salivary gland tissue alone from Ovx-B6 mice. α-tubulin protein as internal control was present. Data are representative of five mice in each group.

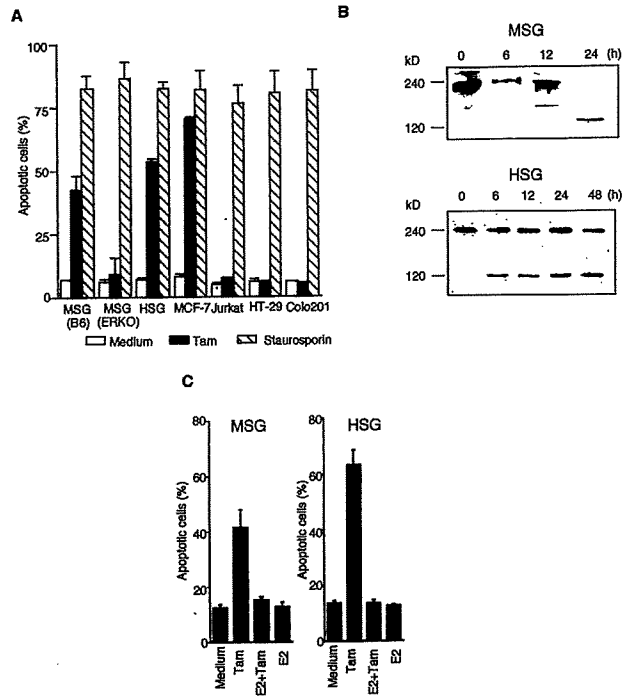


Figure 3. A: Effect of Tam (1×10^{-7} M) on apoptosis in various mouse and human cells (MSG, HSG, MCF-7, Jurkat, HT-29, and Colo201). Staurosporin (1 μmol/L) was used as common apoptotic reagent. **B:** α-fodrin cleavage into 120 kd in MSG and HSG cells induced by Tam. Western blot analysis with mouse monoclonal Ab to α-fodrin was performed in MSG and HSG cells stimulated with Tam (1×10^{-7} M) for 24 or 48 hours. The data are representative of three individual experiments. **C:** Tam-induced apoptosis of MSG and HSG cells was significantly reduced by the pretreatment with estrogen. Apoptotic cells were detected by flow cytometer using propidium iodide staining and Annexin V-FITC. The data are the mean ± SD from three individual experiments.

that antiestrogenic action to the epithelial tissues seems to be transient *in vivo*.

Effect of Estrogen Deficiency on α-Fodrin Proteolysis

We next investigated whether estrogen deficiency is involved in the formation of pathogenic autoantigens in salivary glands. To analyze α-fodrin proteolysis in the salivary glands from Ovx-B6 mice, Western blot analysis was performed using tissue samples. Although an intense band expressing 120-kd α-fodrin was found in the salivary gland samples from Ovx-B6 mice, no cleavage products of α-fodrin were observed in other organs from Ovx mice or any of the samples from Sham mice, while expression of α-tubulin as a control was present (Figure 2). Thus, estrogen deficiency induces *in vivo* proteolysis of α-fodrin in association with apoptosis in the normal salivary gland cells.

Tamoxifen (Tam)-Induced Apoptosis and α-Fodrin Cleavage in Salivary Gland Cells

It has been reported that the antiestrogen tamoxifen (Tam) induces cell death in the human breast cancer cell line MCF-7.[32] To examine whether Tam induces apoptosis in the mouse and human salivary gland (MSG and HSG) cells, the cells were treated with 1×10^{-10} to 1×10^{-6} (M) Tam for 48 hours. We found a time- and concentration-dependent increase in number of apoptotic MSG and HSG cells until 48 hours (data not shown). In contrast to the colon cancer cells (HT-29 and Colo201) or Jurkat cells, apoptosis was induced in MSG, HSG, and MCF7 cells treated with Tam (Figure 3A). Of importance is that the 240-kd α-fodrin in Tam-induced apoptotic MSG and HSG cells was cleaved into 120-kd fragment in a time-dependent manner on Western blotting (Figure 3B). In Tam-induced apoptotic MCF7 cells, negligible levels of cleaved products of α-fodrin were found on Western blotting. We next examined whether estrogen could inhibit Tam-induced apoptosis of MSG and HSG cells. As shown in Figure 3C, Tam-induced apoptosis of MSG and HSG cells was significantly reduced by the pretreatment with estrogen. These data indicate that apoptosis of mouse and human salivary gland cells followed by α-fodrin cleavage into 120-kd fragment could be induced by an antiestrogenic action.

Participation of Caspases in Estrogen-Deficient Salivary Gland Cells

We next investigated whether apoptotic proteases are involved in the α-fodrin cleavage during Tam-induced apoptosis in MSG and HSG cells. A significant increase in caspase-1 activity was detected with relatively elevated caspase-3 and -8 activity on Tam-induced apopto-

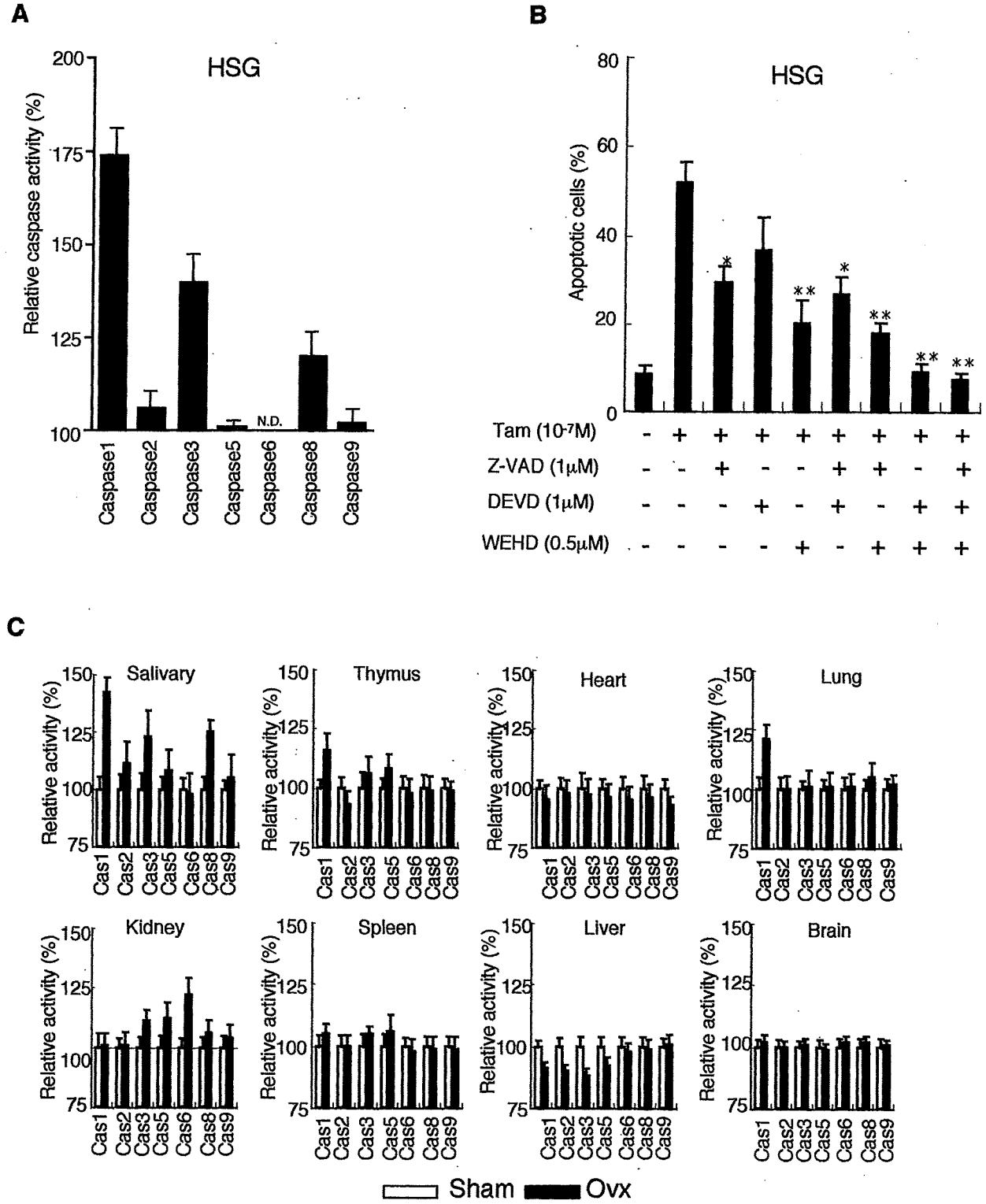


Figure 4. A: A significant increase in caspase-1 activity with relatively elevated caspase-3, and caspase-8 activity on Tam-induced apoptosis in HSG cells. **B:** Tam-induced apoptosis in HSG cells was inhibited considerably by the treatment with caspase inhibitors zVAD, DEVD, and WEHD. **C:** A significantly elevated caspase-1 activity in the salivary gland tissues is observed in Ovx-B6 mice with slight elevation of caspase-3, and caspase-8 activity. No significant differences in caspase activities were observed in other organs.

tic HSG cells (Figure 4A). In addition, Tam-induced apoptosis in HSG cells was inhibited considerably by treatment with caspase inhibitors zVAD, DEVD, and WEHD (Figure 4B). We next examined the level of the caspase activities (caspase-1, -2, -3, -5, -6, -8, and -9) in various organs from Ovx- and non-Ovx-B6 mice. Figure 4C shows that a significantly elevated caspase-1 activity in the salivary gland tissues is observed in Ovx-B6 mice,

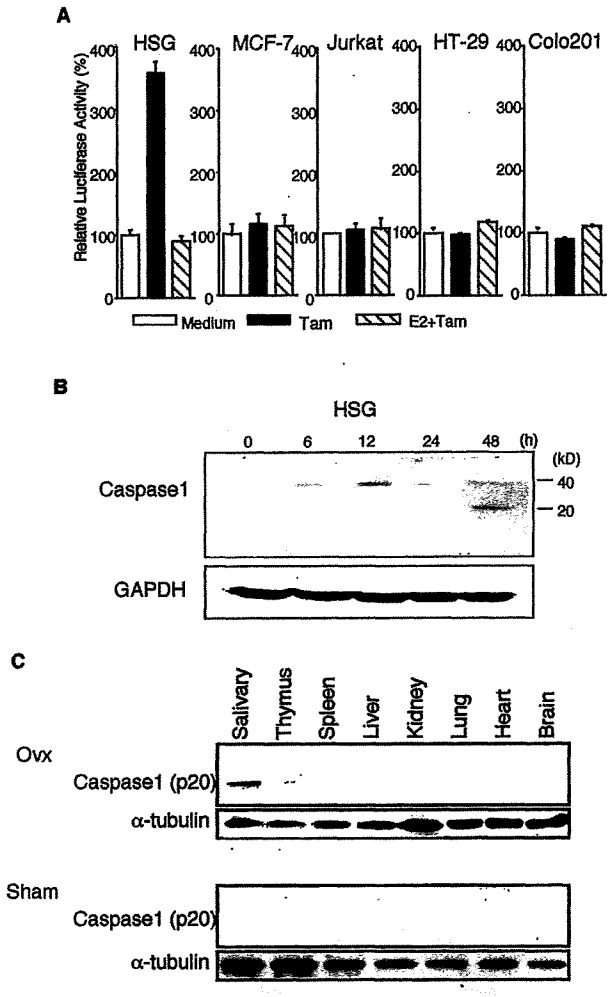


Figure 5. A: Luciferase assay of caspase-1 promoter activities in HSG cells stimulated with Tam. HSG, MCF-7, Jurkat, HT-29, and colo201 cells, after transfection with caspase-1 promoter Luc construct, were stimulated for 2 hours without or with 1×10^{-7} M Tam. Estrogen (17β-estradiol, 1×10^{-8} M) was added to the cells 12 hours before Tam stimulation. The results are the mean values of three independent experiments run in triplicate. **B:** Western blot analysis of caspase-1 in apoptotic HSG cells stimulated with Tam, showing an increase in procaspase-1 (40 kd) at 12 hours, and a time-dependent increase in caspase-1 active form (p20). Cytosolic extracts were prepared from HSG cells which were treated with Tam (1×10^{-7} M) for various times. **C:** Detection of caspase-1 active form (p20) in the salivary gland tissue, but not in various organs from Ovx- and Sham-B6 mice on Western blot analysis. α-tubulin proteins were used as internal control. Data were representative in triplicate.

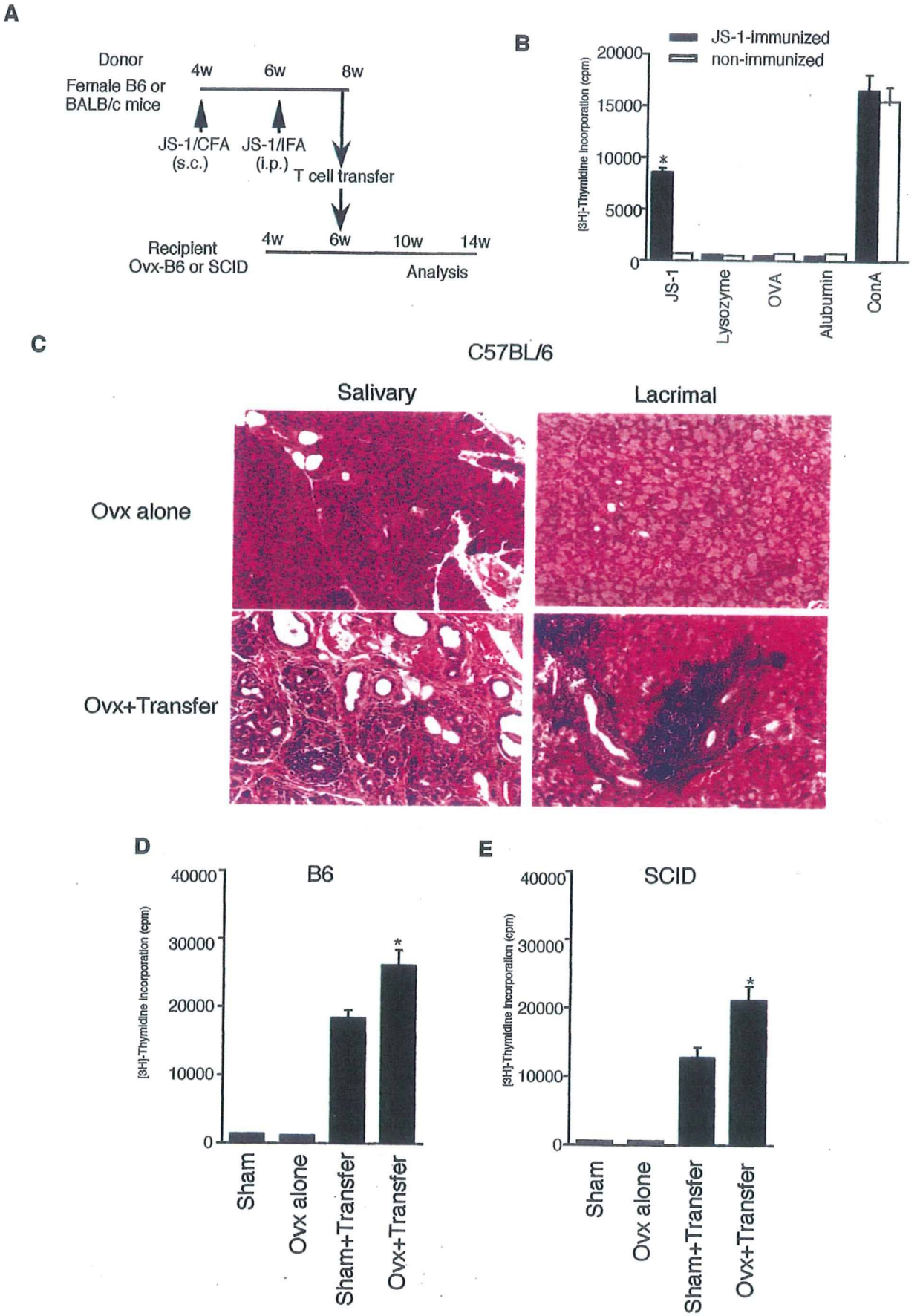
with slight elevation of caspase-3 and 8 activity. No differences in caspase activities were observed in other organs. These results suggest that estrogen deficiency stimulates caspase activity, especially caspase-1, in the salivary gland tissues *in vivo*. We then examined whether Tam could influence the promoter activity of caspase-1 in HSG cells. After transfection with the plasmid containing the caspase-1 promoter-ligated upstream of the luciferase gene, HSG cells were stimulated with Tam, and then luciferase assay was performed. Figure 5A shows an increased promoter activity of caspase-1 after stimulation with Tam in HSG cells, but not in MCF-7, Jurkat, HT-29, and colo201 cells. The increase in caspase-1 promoter activity observed in HSG cells was significantly reduced by the addition of estrogen. We observed a time-dependent expression of caspase-1 (p20) activities until 48 hours on Western blot analysis (Figure 5B). Figure 5C shows that a distinct expression of caspase-1 (p20) in the salivary gland tissues is observed in Ovx-B6 mice. These results suggest that the salivary gland cell apoptosis could be induced by caspase activation, especially caspase-1, in estrogen deficient state.

Adoptive Transfer of α-Fodrin-Reactive T Cells into Ovx-B6 and -SCID Mice

We examined the adoptive transfer experiments using α-fodrin-reactive T cells into Ovx-B6 mice as shown in protocol (Figure 6A). Before the cell transfer, we confirmed the proliferative T-cell response against JS-1, but not against lysozyme, albumin, and ovalbumin, of JS-1-immunized mice (Figure 6B). Consequently, inflammatory lesions developed exclusively in the salivary and lacrimal gland at 4 and 8 weeks after the transfer with 5×10^6 α-fodrin-reactive T cells, not with 5×10^6 OVA-reactive T cells, while no inflammatory lesions in any other organs were detectable (Table 1 and Figure 6C). When 5×10^6 α-fodrin-reactive $CD4^+$ or $CD8^+$ fractionated T cells were transferred into Ovx-B6 and Ovx-SCID mice, no inflammatory lesions were observed in any organs (data not shown). Proliferative T-cell response against recombinant α-fodrin (JS-1) was clearly observed in spleen cells from transferred Ovx-B6 mice (Figure 6D). In Ovx-SCID mice, inflammatory lesions were also induced in the salivary and lacrimal gland at 8 weeks after the transfer with 5×10^6 α-fodrin-reactive T cells, while no inflammatory lesions in any other organs were detectable (Table 1). Significant proliferative T-cell response against recombinant α-fodrin (JS-1) was observed in spleen cells from transferred Ovx-SCID mice (Figure 6E).

Discussion

The mechanisms responsible for the development of autoimmune diseases during the postmenopausal stage are still unclear. Previous reports concerning gender differences in autoimmunity have suggested that estrogen influences the cytokine production of effector cells and autoantibody production.[33,34] The distinct immune environments in males and females underlie many of the gender-related differences in autoimmunity. These environments are established by the cytokines that are released by immune cells, particularly T helper (Th) lymphocytes. Sex hormones, pituitary hormones including prolactin, and growth hormones, as well as liver-derived insulin-like growth factor-1 affect autoimmune diseases by modulating cytokine productions.[35,36] Women have higher levels of these hormones than men. Estrogen withdrawal after menopause leads to an increase in the production of cytokines, such as granulocyte-macrophage colony-stimulating factor, IL-1, IL-6, and TNF-α.[37] Although many studies have described the effects of estrogen on cytokine productions in effector cells, much less is known about the effect of estrogen deficiency in


A
Donor
Female B6 or
BALB/c mice
4w
6w
8w
JS-1/CFA
(s.c.)
JS-1/IFA
(i.p.)
T cell transfer
Recipient
Ovx-B6 or SCID
4w
6w
10w
14w
Analysis
B
JS-1-immunized
non-immunized
[3H]-Thymidine incorporation (cpm)
20000
15000
10000
5000
0
*
JS-1
Lysozyme
OVA
Albumin
ConA
C
C57BL/6
Salivary
Lacrimal
Ovx alone
Ovx+Transfer
D
B6
[3H]-Thymidine incorporation (cpm)
40000
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40000
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Ovx alone
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Ovx+Transfer

Table 1. Frequency of Inflammatory Lesions in the Salivary and Lacrimal Glands in Transferred C57BL/6 and SCID Mice Treated with Ovx

Treatment	No. of mice	No. of mice with lesions*		
		Submandibular	Parotid	Lacrimal
4 weeks after transfer (C57BL/6)				
Ovx alone†	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer‡	7	5/7	7/7	6/7
Sham+transfer	7	0/7	0/7	0/7
Ovx+transfer (OVA)§	5	0/5	0/5	0/5
8 weeks after transfer (C57BL/6)				
Ovx alone†	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer§	7	6/7	7/7	7/7
Sham+transfer	7	0/7	0/7	0/7
Ovx+transfer (OVA)§	5	0/5	0/5	0/5
8 weeks after transfer (SCID)				
Ovx alone†	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer‡	7	5/7	6/7	6/7
Sham+transfer	7	0/7	0/7	0/7

*Histological evaluation for frequency of inflammatory lesions in the salivary and lacrimal glands was done according to the method proposed by White and Casarett.[30]

†Female C57BL/6 (B6) and SCID mice were ovariectomized (Ovx) at 4 weeks of age.

‡Ovx-B6 and SCID mice were transferred intraperitoneally with 5×10^6 α-fodrin-reactive T cells at 6 weeks of age.

§Ovx-B6 mice were transferred with 5×10^6 OVA-reactive T cells. α-fodrin-reactive T cells were obtained from B6 or BALB/c mice as described in detail in the text.

target organs of postmenopausal women. Thus, it is required to determine how estrogen deficiency influences the expression of autoantigens in target cells before the infiltration of lymphocytes into the salivary glands.

In this study, we have demonstrated a significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen deficient healthy B6 mice. Moreover, inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α-fodrin-reactive T cells in both Ovx-B6 and Ovx-SCID mice. These data indicate that α-fodrin cleavage triggered by estrogen deficiency plays a role in the development of autoimmune exocrinopathy in the salivary and lacrimal gland. By contrast, apoptotic cells in the salivary glands were not found in ERαKO mice. In *in vitro* studies using primary cultured MSG and HSG, we found a cleavage product of 120-kd α-fodrin in cells that had undergone Tam-induced apoptosis, not in other type of cells including MCF-7. Since pretreatment with estrogen inhibits the Tam-induced apoptosis of MSG and HSG cells, estrogen may play a crucial role in the apoptosis-related signal pathway. When we analyzed whether cystein proteases are involved in Tam-induced apoptosis of HSG cells, we observed a time-dependent increase in the active forms of caspase-1. In addition, we found that the promoter activity of caspase-1 was significantly increased when HSG cells transfected with the promoter-caspase-1 gene were stimulated with Tam. Indeed, active forms of caspase-1 was detected only in the salivary gland tissues from Ovx-B6 mice. It has been reported that estrogen inhibits the apoptosis of human myocytes and endothelial cells by reducing the activity of caspase-1-like protease.[25,26] On the other hand, estrogen has been shown to inhibit bone resorption by directly inducing apoptosis of bone-resorbing osteoclasts.[38] Our results suggest that antiestrogenic actions might induce the salivary gland apoptosis through a caspase-1-mediated pathway. It is assumed that estrogen acts as a negative regulator of caspase-1 activity in the salivary gland cells.

When MSG and HSG cells were induced to undergo apoptosis using Tam, the 240-kd α-fodrin was cleaved into a single detectable fragment of 120 kd. Among the substrates cleaved during apoptosis are nuclear autoantigen targets for systemic autoimmune diseases such as PARP, U1–70-kd, the nuclear lamin, and DNA-dependent kinase.[39–43] In organ-specific autoimmune diseases, no evidence in *in vivo* cleavage of self proteins during apoptosis has been demonstrated. Our data suggest that antiestrogenic actions have a potent effect on the proteolysis of α-fodrin autoantigen in the salivary gland through up-regulation of caspase-1 activity. These results strongly suggest that α-fodrin fragments induced by Ovx may play an important role on the development of autoimmune lesions as a pathogenic autoantigen. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are further investigated.

In conclusion, we have demonstrated that antiestrogenic actions, including estrogen deficiency or Tam stimulation, may have a crucial influence on apoptosis and

Figure 6. A: Experimental protocol of adoptive transfer using B6 and SCID mice as indicated. **B:** Significant proliferative response of the splenic T cells against recombinant α-fodrin (JS-1, 10 μg/ml) were confirmed before the transfer (*$P < 0.01$, Student's *t*-test). Antigen-stimulated blastogenesis was measured in spleen T cells from immunized and non-immunized mice. No proliferative responses were found to lysozyme (5 μg/ml), albumin (5 μg/ml), and OVA (5 μg/ml). Data are expressed as counts per minute ± SD in triplicate. **C:** Representative histological features of the salivary and lacrimal glands in Ovx+transfer, and Ovx-alone mice (hematoxylin and eosin, magnification, ×120). **D:** Significant proliferative response of the splenic T cells against recombinant α-fodrin (JS-1, 10 μg/ml) in Ovx+transfer B6 mice was observed (*$P < 0.01$, Student's *t*-test). **E:** Significant proliferative response of the splenic T cells against recombinant α-fodrin (JS-1, 10 μg/ml) in Ovx+transfer SCID mice was observed (*$P < 0.05$, Student's *t*-test). Data are expressed as counts per minute ± SD in triplicate.

α-fodrin proteolysis through an increased caspase activity in the salivary gland cells, suggesting a novel mechanism for the development of organ-specific autoimmunity in postmenopausal women.

References

1. Lahita RG: The role of sex hormones in systemic lupus erythematosus. Curr Opin Rheumatol 1999, 11:352–356
2. Bateman A, Singh A, Kral T, Solomon S: The immune-hypothalamic-pituitary-adrenal axis. Endocrinol Rev 1989, 10:92–112
3. Grossman C: Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. J Steroid Biochem 1989, 34:241–251
4. Mooradian A, Morley J, Korenman S: Biological actions of androgens. Endocrinol Rev 1987, 8:1–28
5. Keisler LW, Vom-Saal FS, Keisler DH, Walker SE: Hormonal manipulation of the prenatal environment alters reproductive morphology and increases longevity in autoimmune NZB/W mice. Biol Reprod 1991, 44:707–716
6. Carlsten H, Nilsson N, Jonsson R, Backman K, Holmdahl R, Tarkowski A: Estrogen accelerates immune complex glomerulonephritis but ameliorates T cell-mediated vasculitis and sialadenitis in autoimmune MRL lpr/lpr mice. Cell Immunol 1992, 144:190–202
7. Jansson L, Olsson T, Holmdahl R: Estrogen induces a potent suppression of experimental autoimmune encephalomyelitis and collagen-induced arthritis in mice. J Neuroimmunol 1994, 53:203–207
8. Hawkins T, Gala R, Dunbar J: The effect of neonatal sex hormone manipulation on the incidence of diabetes in nonobese diabetic mice. Proc Soc Exp Biol Med 1993, 202:201–205
9. Jansson L, Mattsson A, Mattsson R, Holmdah IR: Estrogen-induced suppression of collagen arthritis: V: physiological level of estrogen in DBA/1 mice is therapeutic on established arthritis, suppresses anti-type II collagen T-cell dependent immunity and stimulates polyclonal B-cell activity. J Autoimmun 1990, 3:257–270
10. Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T, Ikegami S: Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. Endocrinol 1999, 140:1893–1900
11. Masuzawa T, Miyaura C, Onoe Y, Kusano K, Ohta H, Nozawa S, Suda T: Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. J Clin Invest 1994, 94:1090–1097
12. Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, Ciliberto G, Rodan GA, Costantini F: Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. EMBO J 1994, 13:1189–1196
13. Ishimaru N, Saegusa K, Yanagi K, Haneji N, Saito I, Hayashi Y: Estrogen deficiency accelerates autoimmune exocrinopathy in murine Sjogren's syndrome through Fas-mediated apoptosis. Am J Pathol 1999, 155:173–181
14. Mayes M: Epidemiologic studies of environmental agents and systemic autoimmune diseases. Environ Health Perspect 1999, 107:743–748
15. Zhao Z, Granucci F, Yeh L, Schaffer P, Cantor H: Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. Science 1998, 279:1344–1347
16. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, Noji S, Sugino H, Hayashi Y: Identification of α-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science 1997, 276:604–607
17. Saegusa K, Ishimaru N, Yanagi K, Mishima K, Arakaki R, Suda T, Saito I, Hayashi Y: Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjögren's syndrome. J Immunol 2002, 169:1050–1057
18. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J: Human ICE/CED-3 protease nomenclature (letter). Cell 1996, 87:171
19. Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR: Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J Biol Chem 1995, 270:6425–6428
20. Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR: Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J Biol Chem 1996, 271:28753–28756
21. Janicke RU, Sprengart ML, Wati MR, Porter AG: Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 1998, 273:9357–9360
22. Cryns VL, Bergeron L, Zhu H, Li H, Yuan J: Specific cleavage of alpha-fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1β-converting enzyme/Ced-3 protease distinct from the poly(ADP-ribose) polymerase protease. J Biol Chem 1996, 271:31277–31282
23. Wang KK, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, Glantz SB, Morrow JS: Simultaneous degradation of αII- and βII-spectrin by caspase-3 (CPP32) in apoptotic cells. J Biol Chem 1998, 273:22490–22497
24. Szende B, Romics I, Vass L: Apoptosis in prostate cancer after hormonal treatment (letter). Lancet 1993, 342:1422
25. Spyridopoulos I, Sullivan A, Kearney M, Isner J, Losordo D: Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. Circulation 1997, 95:1505–1514
26. Pelzer T, Schumann M, Neumann M, DeJager T, Stimpel M, Serfling E, Neyses L: 17β-estradiol prevents programmed cell death in cardiac myocytes. Biochem Biophys Res Commun 2000, 268:192–200
27. Pike CJ: Estrogen modulates neuronal Bcl-xL expression and β-amyloid-induced apoptosis: relevance to Alzheimer's disease. J Neurochem 1999, 72:1552–1563
28. Acarturk F, Robinson JR: Vaginal permeability and enzymatic activity studies in normal and ovariectomized rabbits. Pharm Res 1996, 13: 779–783
29. Oner P, Bekpinar S, Cinar F, Argun A: Relationship of some endogenous sex steroid hormones to leukocyte arylsulphatase A activities in pre- and postmenopausal healthy women. Horm Metab Res 1994, 26:301–304
30. White SC, Casarett GW: Induction of experimental autoallergic sialadenitis. J Immunol 1974, 112:178–185
31. Shirasuna K, Sato M, Miyazaki T: A neoplastic epithelial duct cell line established form an irradiated human salivary gland. Cancer 1981, 48:745–752
32. Kyprianou N, English HF, Davidson NE, Isaacs JT: Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 1991, 51:162–166
33. Ansar-Ahmed S, Penhale WJ, Talal N: Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol 1985, 121:531–551
34. Ansar-Ahmed S, Dauphinee M, Montoya A, Talal N: Estrogen induces normal murine $CD5^+$ B cells to produce autoantibodies. J Immunol 1989, 142:2647–2653
35. Luca IC: Prolactin secretion and the immune system. Rev Med Chir Soc Med Nat Iasi 1997, 101:56–59
36. Whitacre CC, Reingold SC, O'Looney PA: A gender gap in autoimmunity. Science 1999, 283:1277–1278
37. Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R: Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. J Biol Chem 1996, 271:28890–28897
38. Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang N, Hakeda Y, Kumegawa M: Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J Exp Med 1997, 186:489–495
39. Casciola-Rosen L, Nicholson DW, Chong T, Rowan KR, Thornberry NA, Miller DK, Rosen A: Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J Exp Med 1996, 183:1957–1964
40. Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M: Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. J Cell Biol 1994, 126:827–837
41. Casciola-Rosen LA, Anhalt GA, Rosen A: DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. J Exp Med 1995, 182:1625–1634
42. Tu S, Cerlone RA: Cdc42 is a substrate for caspases and influences Fas-induced apoptosis. J Biol Chem 2001, 276:19656–19633
43. Slee EA, Adrain C, Martin SJ: Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem 2001, 276:7320–7326

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Development of Autoimmune Exocrinopathy Resembling Sjögren's Syndrome in Adoptively Transferred Mice With Autoreactive CD4+ T Cells

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Objective. **The pathologic mechanisms responsible for organ-specific tissue damage in primary Sjögren's syndrome (SS) remain unclear, but it has been suggested that the pathology is mediated by autoreactive CD4+ T cells infiltrating the salivary and lacrimal glands. This study was undertaken to investigate whether α-fodrin autoantigen–specific autoreactive CD4+ T cells are capable of inducing autoimmune lesions.**

Methods. **A total of 45 synthetic α-fodrin peptides designed to be 20 amino acid residues in length were generated. To establish an autoreactive T cell line, limiting dilution analysis (LDA) was performed on lymph node cells (LNCs) in the presence of α-fodrin peptides. The effects of adoptive transfer of autoreactive CD4+ T cells into normal syngeneic recipients were investigated.**

Results. **Autoreactive CD4+ T cell lines that recognize synthetic α-fodrin peptide, which produced Th1 cytokines and showed cytotoxic activities, were established in a murine model for SS. T cell receptor V_β usage and third complementarity-determining region (CDR3) sequences indicated that in some cases V_β6-CDR3 genes matched between the tissue-infiltrating T cells and the autoreactive T cell lines. Adoptive transfer of the autoreactive CD4+ T cells into normal syngeneic recipients induced autoimmune lesions quite similar to those of SS.**

Conclusion. **Our data help to elucidate the pathogenic mechanisms responsible for tissue destruction in autoimmune exocrinopathy and indicate that autoreactive CD4+ T cells play a pivotal role in the development of murine SS.**

Primary Sjögren's syndrome (SS) is a T cell–mediated autoimmune disease, and autoreactive T cells bearing the CD4 molecule may recognize unknown self antigen, triggering autoimmunity in the salivary and lacrimal glands and leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) (1,2). Previously, we identified involvement of a 120-kd α-fodrin autoantigen in the pathogenesis of primary SS in humans and rodents (3,4), but the mechanisms for tissue destruction in target organs remain unclear.

Although an important role for T cells in the development of organ-sepcific autoimmune disease has been suggested, it is not known whether disease is initiated by a restricted inflammatory reaction to an organ-specific autoantigen. In most cases, antigenic challenge results in the establishment of immunologic memory, a state in which the immune system is maintained to respond effectively upon recurrent antigenic exposure. Autoreactive T cells generally respond to a limited number of immunodominant epitopes in self antigenic proteins, including myelin basic protein, thyroglobulin, and glutamic acid decarboxylase (5–8). Thymectomy on day 3 after birth (3d-Tx) is followed by the development of organ-specific autoimmune diseases (9,10). The *sld* mutation in NFS/N mice (NFS/*sld*, H-2D^q) is involved in the mucous cell differentiation of the sublingual gland (11). Using 3d-Tx NFS/*sld* mutant

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mice, we have established and analyzed a murine model of primary SS in which the animals spontaneously develop a disease with many of the characteristics of human SS (12–14). The T cell receptor (TCR) $V_\beta 8$ and $V_\beta 6$ genes are preferentially used in these lesions from the onset of disease in the 3d-Tx NFS/*sld* mouse model (12). However, little is known about the events triggering T cell invasion of the the salivary and lacrimal glands in prelude to the development of autoimmune lesions.

Alpha-fodrin is an actin-binding protein that is found at the periphery of chromaffin cells and may be involved in secretion (15). The stimulation of secretion in parotid acinar cells is associated with dramatic rearrangements of the sub-plasmalemmal cytoskelton of α-fodrin (16). In the present study we established α-fodrin–reactive T cell lines capable of inducing autoimmune lesions similar to those found in SS. Moreover, the TCR V_β usage and the third complementarity-determining region (CDR3) sequences of the autoreactive T cell lines were determined.

MATERIALS AND METHODS

Mice. NFS/N mice carrying the mutant gene *sld* (11) were raised in our specific pathogen–free mouse colony. Thymectomy was performed on day 3 after birth.

Histologic and immunohistologic analysis. All organs were removed from the mice, and the sections were stained with hematoxylin and eosin. Histologic grading of the inflammatory lesions was done determined as described previously (17). Immunohistologic analysis was performed by the avidin–biotin–immunoperoxidase method utilizing ABC reagent (Vector, Burlingame, CA). Monoclonal antibodies used were as follows: biotinylated rat monoclonal antibodies to CD4 and CD8 (Cedarlane, Hornby, Ontario, Canada), Mac-1 (Becton Dickinson, Burlingame, CA), and B220, $V_\beta 8$, $V_\beta 6$, interleukin-2 (IL-2), IL-4, and interferon-γ (IFNγ) (all from PharMingen, San Diego, CA).

Recombinant α-fodrin autoantigen. Recombinant α-fodrin protein, the complementary DNA (cDNA) encoding human α-fodrin (JS-1: 1–1784 bp, 2.7A: 2258–4884 bp, 3′DA: 3963–7083 bp) (18) was constructed by inserting cDNA into the *Eco* RI site of pGEX-4T 1, 2, and 3. The mouse sequences of α-fodrin are identical to the human sequences.

Assessment of proliferative T cell response. Single cell suspensions of spleen cells and lymph node cells (LNCs) were cultured in 96-well flat-bottomed microtiter plates (5×10^5 cells/well) in RPMI 1640 containing 10% fetal calf serum (FCS), penicillin/streptomycin, and β-mercaptoethanol. Cells were cultured with each recombinant α-fodrin protein (JS-1, 2.7A, and 3′DA) (5 μg/ml). During the last 8 hours of the 72-hour culture period, 1 μCi of ^{3}H-thymidine was added per well, and the incorporated radioactivity was determined using an automated beta liquid scintillation counter. We isolated tissue-infiltrating mononuclear cells from affected salivary glands as described previously (14). Infiltrating T cells were purified using nylon wool (Wako, Osaka, Japan).

TCR V_β usage and CDR3 sequencing of polymerase chain reaction (PCR) products. To investigate the comparison of clonotypes of infiltrating T cells in vivo and autoreactive T cell lines, reverse transcriptase PCR (RT-PCR) was used to discriminate the diversities in the D, J, and N regions. Total RNA was prepared with Isogen (Nippon Gene, Tokyo, Japan), and amplification was performed with *Taq* polymerase with 5′ primer specific for the TCR $V_\beta 6$ and $V_\beta 8$ genes and a 3′ primer specific for the TCR C_β gene. The diluted sample (2 μl) was electrophoresed in nondenaturing 5% polyacrylamide gels containing 10% glycerol. After electrophoresis, the DNA was transferred to Immobilon-S (Millipore, Intertech, Bedford, MA) and hybridized with biotinylated C_β probe, streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Millipore Intertech). The inserted TCR genes were sequenced with dye-labeled primers and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), using a 373A automated DNA sequencing system (Applied Biosystems).

Synthetic peptides. Peptides were synthesized using TBOC chemistry with a model 430A peptide synthesizer (Applied Biosystems, CA). A total of 45 synthetic peptides designed to be 20 amino acid residues in length, overlapping by 5 amino acid residues, were generated.

Autoreactive T cell line. To establish an α-fodrin peptide–specific T cell line, limiting dilution analysis (LDA) was performed as described previously (19,20), on LNCs in the presence of α-fodrin peptides and irradiated syngeneic spleen cells. LNCs from 3d-Tx NFS/*sld* mice (5 weeks old) were cultured with α-fodrin peptides (10 μg/ml) in RPMI 1640 supplemented with 10% FCS, 10 m*M* HEPES, and 100 units (100 μg/ml) 100 penicillin/streptomycin in 96-well plates at 1×10^6 cells/well. On day 3, IL-2 (Genzyme, Cambridge, MA) was added; cells were fed with media containing 0.5 ng/ml IL-2 every 3 days. On day 14, an aliquot was analyzed for reactivity to α-fodrin peptides. Cells (1×10^4) from each cell line were cocultured with 1×10^4 irradiated autologous splenocytes in duplicate for 72 hours. Alpha-fodrin–specific T cell lines (stimulation index >3) were retested using synthetic peptides and were maintained by restimulation at 10–14–day intervals with α-fodrin peptide pulse-irradiated splenocytes. Following a third round of stimulation, LDA revealed cloned T cell lines. Autoreactive T cell lines were maintained by stimulation with IL-2 and feeder cells at 7–10–day intervals.

Flow cytometric analysis. Single cell suspensions were stained with antibodies conjugated to phycoerythrin (PE) (anti-CD3 [Gibco BRL, Grand Island, NY]; anti-CD4, B220) or anti-CD4; fluorescein isothiocyanate (FITC) (anti-CD8, Thy1.2, anti-CD44, anti-CD45RB, anti–Mel-14 [the latter 4 from PharMingen]), and analyzed on an EPICS counter (Coulter, Hialeah, FL). For analysis of intracellular cytokines by flow cytometry, cells (10^6/ml) were activated with immobilized anti-CD3 monoclonal antibody (Cedarlane) for 4 hours. Monensin (Wako) was added at 2 m*M*, and 2 hours later cells were collected, washed, and permeabilized with 0.1% saponin in phosphate buffered saline at 4°C for 10 minutes. Cells were incubated with FITC-conjugated anti-IL-2 (8 μg/ml), PE-conjugated anti–IL-4 (5 μg/ml), and FITC-conjugated anti-IFNγ (1 μg/ml) and analyzed on an EPICS counter.

Cytotoxicity assay. Cytotoxicity assays were performed as described previously (21), using peptide-pulsed (10 μg/ml) mouse salivary gland (MSG) cells labeled with ^{51}Cr sodium chromate as target at a 1:50 target:effector ratio.

Cell transfer. To examine whether autoreactive CD4+ T cells induce autoimmune lesions, cells from T cell line 21–1 (1×10^6) were injected intraperitoneally into irradiated (7.5 Gy) normal NFS/*sld* mice at 4 weeks, and analyses performed at 8 weeks (n = 7) and 12 weeks (n = 8) after the injection. As controls, 1×10^6 splenic CD4+ T cells nonpulsed or pulsed and with fibronectin fragment peptide (5 μg/ml; Sigma, St. Louis, MO) from syngeneic mice were injected intraperitoneally into irradiated (7.5 Gy) NFS/*sld* mice and analyzed in the same manner (n = 5 for each).

Measurement of fluid secretion. Measurement of tear and saliva volume in the transferred NFS/*sld* mice was performed by modification of previously described methods (14,21,22).

Western blot analysis. To detect serum autoantibodies against 120-kd α-fodrin antigen (3), samples were solublized by heating, and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The autoantigen was electrotransferred to nitrocellulose, which was then quenched with 1% powdered milk in borate buffered saline. Nitrocellulose membranes were incubated with testing serum at a 1:200 dilution in borate buffered saline, and then incubated with peroxidase-conjugated horse anti-mouse IgG (Vector) at a 1:1,000 dilution.

RESULTS

Establishment of autoreactive T cell lines. We have previously identified a cleavage product of 120-kd α-fodrin as an important autoantigen in the pathogenesis of primary SS in both humans and rodents (3,4). To determine whether an immune response in mice with experimental SS could be mounted against recombinant α-fodrin protein, the cDNA encoding human α-fodrin (JS-1, 2.7A, and 3′DA) were constructed by inserting cDNA into the *Eco* RI site of pGEX-4T 1, 2, and 3. When we compared, in parallel, the proliferative T cell responses with individual recombinant α-fodrin fusion protein, we detected a significantly increased proliferation in SS mouse LNCs, spleen cells, and tissue-infiltrating T cells stimulated with JS-1 protein (N-terminal portion of α-fodrin) (data not shown). By LDA, we succeeded in isolating 3 strongly proliferative autoreactive T cell lines (clones 21–1, 21–2, and 21–3) from JS-1 peptide (p21)–stimulated LNCs (Figure 1A), but not from control peptide–stimulated cells. The majority of autoreactive T cells were CD4+ cells bearing $V_\beta 6$ and containing Th1 cytokines such as IL-2 and IFNγ, but not IL-4 (Figure 1B). We confirmed that the autoreactive CD4+ T cell lines had significant cytotoxicity, against MSG cells from NFS/*sld* mice when tested in a ^{51}Cr-release assay (Figure 1C). In contrast, the autoreactive T cells did not kill major histocompatibility complex (MHC)–matched targets of newborn keratinocytes (data not shown).

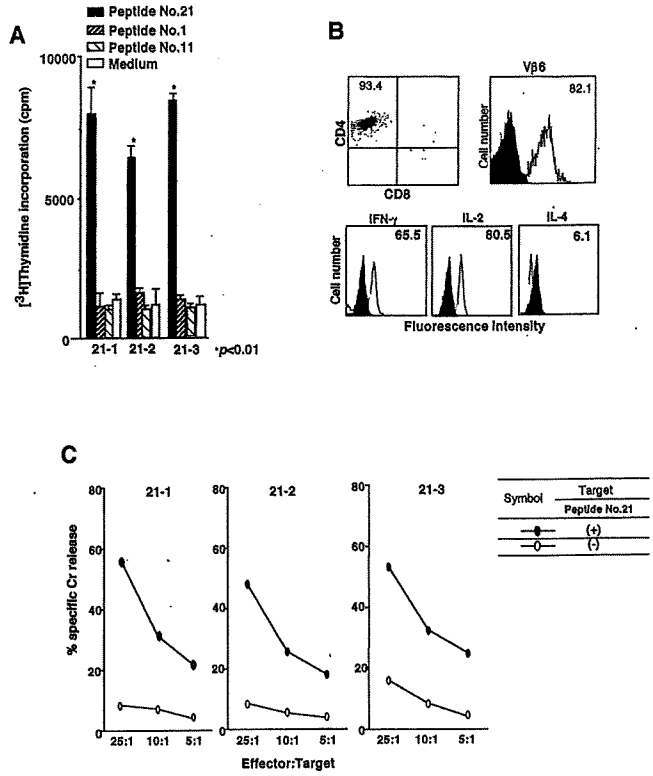

Figure 1. Establishment of autoreactive T cell lines. **A,** Three strongly proliferative T cell lines (21–1, 21–2, 21–3) from JS-1 peptide p21–stimulated lymph node cells were isolated and ^3H-thymidine incorporated measured. Values are the mean and SEM. **B,** Flow cytometric analysis, showing that the majority of these T cells were CD4+ and $V_\beta 6$+ and contained interleukin-2 (IL-2) and interferon-γ (IFNγ), but not IL-4. **C,** Results of ^{51}Cr-release assay, demonstrating that the autoreactive CD4+ T cell lines had significant cytotoxicity when tested against mouse salivary gland cells. Values are the mean from triplicate studies.

The TCR V_β usage and the CDR3 sequences of 3 autoreactive T cell lines were determined by RT-PCR amplification and sequencing of the PCR products. Notably, in some cases these sequences (SISAETL and SMQN) were homologous to V–D–J β sequences of the T cells from affected glands of mice with experimental SS at 8 weeks (Figure 2).

Development of autoimmune lesions after adoptive transfer of autoreactive CD4+ T cells. To analyze whether the autoreactive T cells cause autoimmune lesions, cells from CD4+ T cell line 21–1 (1×10^6) were transferred intraperitoneally into irradiated (7.5 Gy) normal NFS/*sld* mice at 4 weeks. Organ-specific auto-

	Vβ6	N-D-N	Jβ		
T cells infiltrated in salivary gland					
	TGTGCCAGC	AGTTATGCAGAA	TTCGGTCCCGGCACCAGGCTCGGTTTT	2.1	
	C A S	S M Q N	F G P G T R L G F		
	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTTGGTGCGGGCACCCGACTATCGGTG	2.3	
	C A S	S I S A E T L	Y F G A G T R L S V		
Autoreactive T cell lines					Frequency
21-1	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTTGGTGCGGGCACCCGACTATCGGTG	2.3	6/10
	C A S	S I S A E T L	Y F G A G T R L S V		
	TGTGCCAGC	AGTATGACAGTA	TTCGGTCCCGGCACCAGGCTCGGTTTT	2.1	1/10
	C A S	S M T V	F G P G T R L G F		
	TGTGCCAGC	AGTATAGGGGAGAGATATGAACAG	TACTTCGGTCCCGGCACCAGGCTCACTGTT	2.6	1/10
	C A S	S I G E R Y E Q	Y F G P G T R L T V		
	TGTGCCAGC	AGCCCCGCAGAAACGCTG	TATTTTGGCCCCGGNACCAGACTGACTGTT	2.6	1/10
	C A S	S P A E T L	Y F G P X T R L T V		
21-2	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTTGGTGCGGGCACCCGACTATCGGTG	2.3	7/10
	C A S	S I S A E T L	Y F G A G T R L S V		
	TGTGCCAGC	AGTATTTGGGTTGAACAG	TACTTCGGTCCCGGCACCAGGCTCACGGTT	2.5	1/10
	C A S	S I W V E Q	Y F G P G T R L T V		
	TGTGCCAGT	AGTACCGGNCTGGGGGGTTCAAGACACCCAG	TACTTTGGGCCAGGCACTCGGCTCCTCGTG	2.5	1/10
	C A S	S T G L G V Q D T Q	Y F G P G T R L L V		
	TGTGCCAGC	AGCCCGCAGAAACGCTGT	ATTTTGGCCTTGGNACCAGACTGACTGTT	2.6	1/10
	C A S	S P A E T L	Y F G L X T R L T V		
21-3	TGTGCCAGC	AGTTATGCAGAA	TTCGGTCCCGGCACCAGGCTCGGTTTT	2.1	7/10
	C A S	S M Q N	F G P G T R L G F		
	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTTGGTGCGGGCACCCGACTATCGGTG	2.3	1/10
	C A S	S I S A E T L	Y F G A G T R L S V		
	TGTGCCAGT	AGTACCGGNCTGGGGGTTCAAGACACCCAG	TACTTTGGGCCAGGCACTCGGCTCCTCGTG	2.5	1/10
	C A S	S T G L G V Q D T Q	Y F G P G T R L L V		
	TGTGCCAGC	AGTCGGGACGGATATGAACAG	TACTTCGGTCCCGGCACCAGGCTCACGGTT	2.6	1/10
	C A S	S R D G Y E Q	Y F G P G T R L T V		

Figure 2. T cell receptor V_β gene usage and third complementarity-determining region (CDR3) sequences of infiltrating T cells in NFS/*sld* mice that had undergone thymectomy on day 3 after birth and of α-fodrin p21–specific lines (21–2, 21–2, 21–3), determined by reverse transcriptase–polymerase chain reaction amplification and sequencing of the polymerase chain reaction products. The V_β6-CDR3 sequences were homologous between the T cells infiltrating salivary glands and 3 autoreactive T cell lines (underlined).

immune lesions developed exclusively in the salivary and lacrimal glands at 8 weeks (n = 7) and 12 weeks (n = 8) after the injection with autoreactive T cell line 21–1, while a transfer of splenic CD4+ T cells pulsed with fibronectin fragment peptide did not induce any lesions (Figure 3A). Histopathologic examination revealed no inflammatory lesions in other organs including the liver, pancreas, adrenal glands, and reproductive organs of mice treated with the autoreactive T cell line. This suggests that α-fodrin–reactive CD4+ T cells are pathogenic in vivo.

The majority of tissue-infiltrating cells in the salivary and lacrimal glands of mice that underwent adoptive transfer of autoreactive CD4+ T cells were positive for CD4 and V_β6, but not for CD8 or V_β8 (Figure 3B). Very few B220+ B cells were present in inflammatory lesions (results not shown). A large proportion of Th1 cytokine–positive cells (IL-2, IFNγ), but not IL-4–positive cells, was detected in the salivary glands from autoreactive T cell line–treated mice (Figure 4A). Isotype-matched controls were all negative. Moreover, the autoimmune lesions were accompanied by significantly decreased secretion of saliva and tears (Figure 4B). Serum autoantibody production against JS-1 protein could not be detected in adoptively transferred mice (Figure 4C). T cell line–treated mice showed a significant increase of autoantigen (JS-1 and p21)–specific T cell proliferation in the spleen cells, while no responses against ovalbumin or lysozyme were observed (Figure 5A). The activation markers $CD44^{high}$, $CD45RB^{low}$, and $Mel\text{-}14^{low}$ were significantly upregulated in LNCs gated on CD4 from adoptively transferred mice (Figure 5B). Moreover, we found that CD4+ T cells isolated from LNCs of the treated mice had significant cytotoxicity when tested against MSG

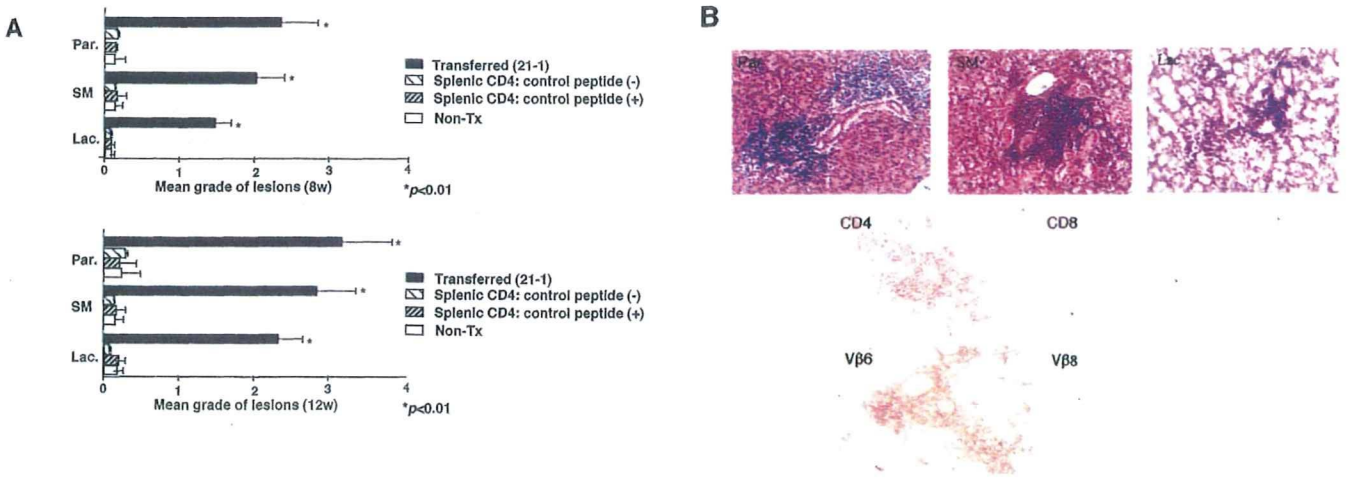

Figure 3. Adoptive transfer of autoreactive T cell line 21–1 into normal syngeneic recipients. **A,** Autoimmune lesions in the salivary and lacrimal (Lac.) glands developed at 8 weeks (n = 7) and 12 weeks (n = 8) after intraperitoneal injection with cells from T cell line 21–1 (1×10^6) into irradiated (7.5 Gy) normal NFS/*sld* mice, but not in controls. Values are the mean and SEM. Par. = parotid gland; SM = submandubular gland; Non-Tx = nonthymectomized. **B,** Representative histologic features in adoptively transferred mice at 12 weeks. The majority of infiltrating lymphocytes were positive for CD4 and V_β6, but not for CD8 or V_β8.

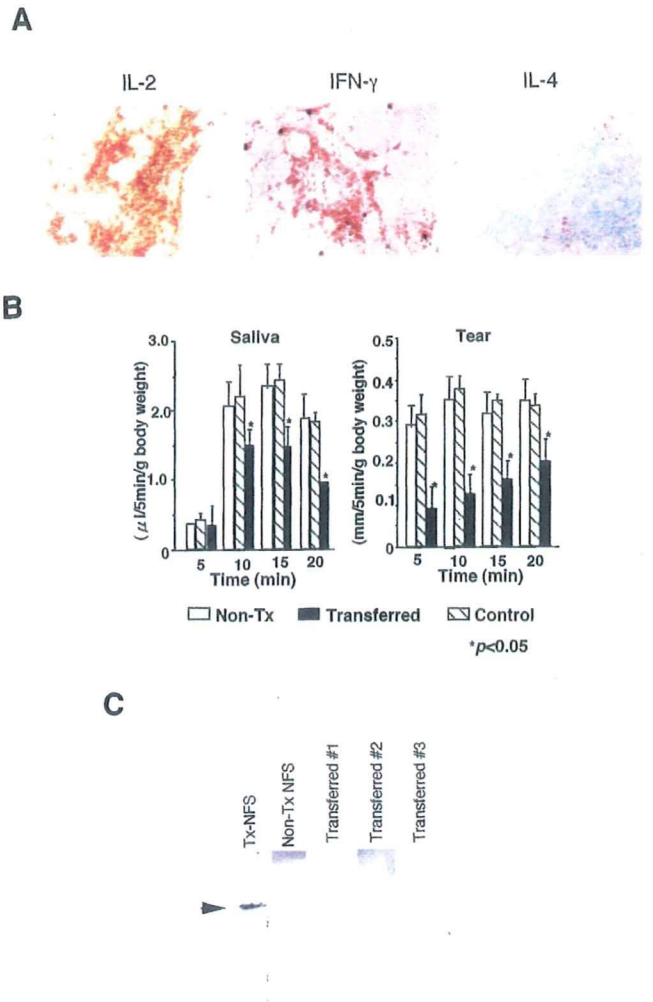

Figure 4. A, Immunohistologic features in adoptively transferred mice. A large proportion of infiltrating cells in the salivary glands were positive for Th1 cytokines such as interleukin-2 (IL-2) and interferon-γ (IFNγ), but not IL-4. **B,** Mean and SEM saliva and tear volume (n = 5 per group) in adoptively transferred mice, control-treated mice, and nonthymectomized (Non-Tx) mice. **C,** Western blot analysis. Production of autoantibodies to 120-kD α-fodrin was not detected in sera from 3 different adoptively transferred mice.

cells from NFS/*sld* mice in a ^{51}Cr-release assay (Figure 5C).

DISCUSSION

Autoreactive T cells are conventionally regarded to be eliminated by negative selection in the thymus or by the induction of peripheral tolerance (23,24). The results described here demonstrate that α-fodrin–reactive CD4+ T cells can induce autoimmune exocrinopathy in normal syngeneic mice. Although the specificity of cytotoxic T lympyocyte (CTL) function has been an important issue in organ-specific autoimmune response, the mechanisms responsible for tissue destruction have not been elucidated. In type 1 diabetes mellitus, the role of environmental factors (25,26), the nature of the initiating inflammatory cell (27,28), and the identity of the inciting antigen(s) (29,30) have all been vigorously debated. We have previously identified a cleavage product of 120-kd α-fodrin as an important autoantigen in the pathogenesis of primary SS in both humans and rodents (3,4). We detected significantly increased proliferation of lymph node cells, spleen cells, and tissue-infiltrating T cells from model mice (3d-Tx NFS/*sld*) stimulated with JS-1 protein (N-terminal portion of α-fodrin).

We succeeded in isolating 3 strongly proliferative autoreactive T cell lines (21–1, 21–2, and 21–3) from JS-1 peptide (p21)–stimulated LNCs. The majority of autoreactive T cells were CD4+ T cells bearing $V_\beta 6$ and containing Th1 cytokines such as IL-2 and IFNγ, but not IL-4. Of importance is that the autoreactive CD4+ T cell lines had significant cytotoxicity when tested against MSG cells in a ^{51}Cr-release assay. Furthermore, the TCR V_β usage and the CDR3 sequences of 3 autoreactive T cell lines were homologous to VDJ_β sequences of the T cells from affected glands of mice with experimental SS. (Figure 2).

Previous studies have suggested that clonally expanded T cell populations with restricted usage of TCR gene segments may be essential for the development of autoimmune diseases including SS (31,32). However, the basis for TCR repertoire selection initiating autoimmunity has not yet been fully understood. It should be noted that in this study, infiltrating T cell sequences that are similar to, and in some cases match, the sequences of the autoreactive T cell lines were found (underlined in Figure 2). Previous work has demonstrated that dual TCR T cells may rescue autoreactive T cells from negative selection in the thymus (33). Our data imply that the established autoreactive T cells are found in the common TCR repertoire ($V_\beta 6$-CDR3: SISAETL). This notion is supported by work by Basu et al (34) which demonstrates the binding of 2 separate ligands, a self peptide (arthritic peptide), and a foreign epitope, on distinct MHC areas by T cells bearing a single TCR.

In the analysis of whether the autoreactive T cells cause autoimmune lesions, we found that organ-specific autoimmune lesions developed exclusively in the salivary and lacrimal glands at 8 and 12 weeks after the intra-

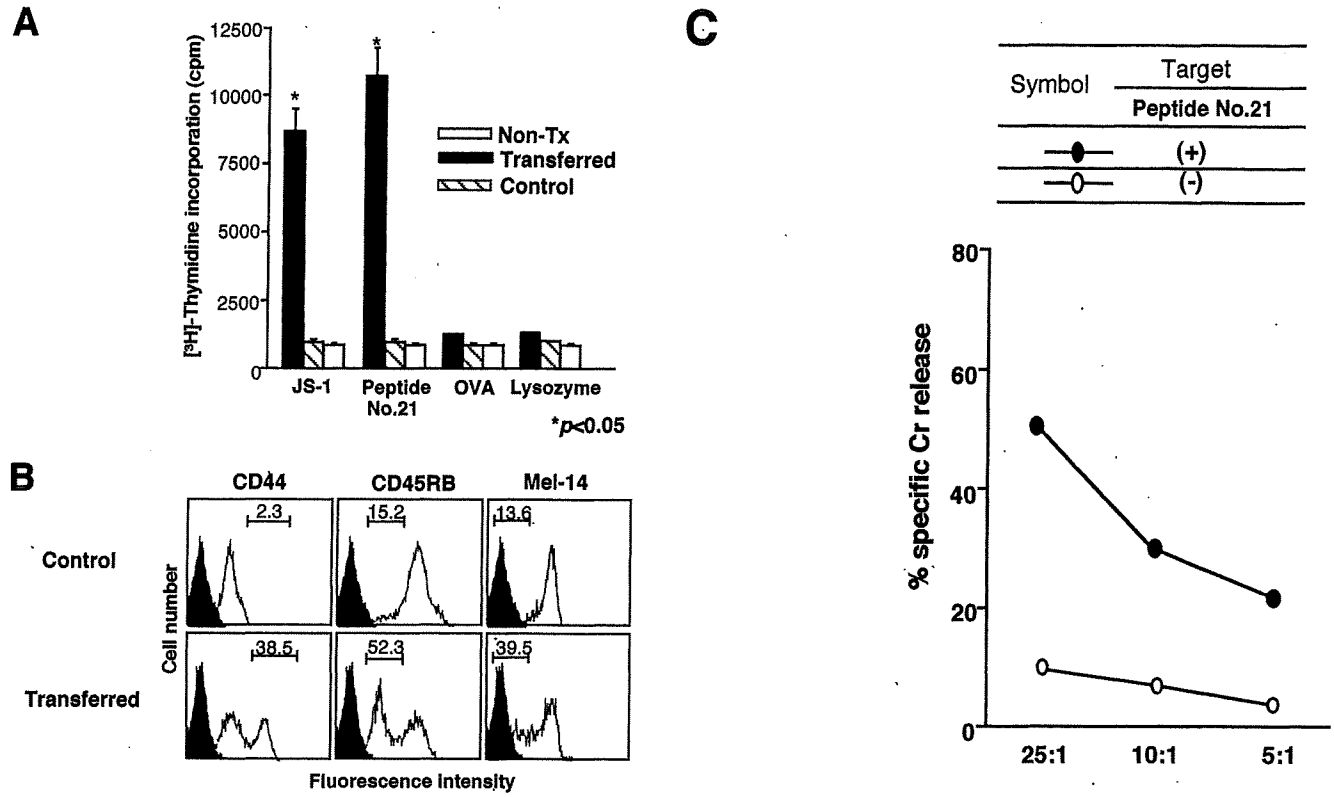

Figure 5. **A,** T cell proliferation in spleen cells. Adoptively transferred mice showed a significant increase of autoantigen (JS-1 and peptide 21)–specific T cell proliferation, while no responses against ovalbumin (OVA) or lysozyme were observed. Values are the mean and SEM of triplicate experiments. Non-Tx = nonthymectomized. **B,** Flow cytometric analysis, showing that the activation markers CD44^{high}, CD45RB^{low}, and Mel-14^{low} were significantly up-regulated in lymph node cells gated on CD4 from adoptively transferred mice. **C,** Results of ^{51}Cr-release assay, demonstrating that CD4+ T cells isolated from lymph node cells of adoptively transferred mice had significant cytotoxicity against salivary gland cells from NFS/*sld* mice. Values are the mean from triplicate studies.

peritoneal injection with autoreactive CD4+ T cells. Adoptively transferred mice showed a significant increase of autoantigen-specific T cell proliferation in the spleen cells, while no responses against ovalbumin or lysozyme were observed. The activation markers were significantly up-regulated in LNCs gated on CD4 from adoptively transferred mice, and CD4+ T cells isolated from LNCs of transferred mice had significant cytotoxicity against MSG cells when tested in a ^{51}Cr-release assay. These data indicate that the autoreactive CD4+ T cells recognizing α-fodrin autopeptide are essentially pathogenic for the development of organ-specific autoimmune lesions in murine SS. Since serum production of autoantibodies against α-fodrin autoantigen could not be detected in transferred mice, it is possible that the adoptively transferred disease in these experiments may be entirely dependent on T cell–mediated immune responses. Thus, a critical autoreactive CD4+ T cell function should be operative in the initial stages of the disease, because the T cells in established lesions show strong proliferative activity and secrete Th1 cytokines. Previous investigations have demonstrated the accumulation of antigen-reactive T cells at the site of the inflammation in several human autoimmune diseases as well as in murine models of human autoimmune diseases (35,36).

In conclusion, we have demonstrated that α-fodrin–specific autoreactive CD4+ T cell lines can be established from α-fodrin peptide p21–stimulated LNCs, and that the autoreactive T cells have significant cytotoxicity against MSG cells when tested in a CTL assay. Moreover, we confirmed the development of autoimmune lesions, quite similar to those found in SS, into normal syngeneic recipients, using autoreactive CD4+ T cells. The results of this study provide evidence of an essential role for autoreactive CD4+ T cells specific for a self peptide in the development of organ-specific autoimmune disease in SS.

REFERENCES

1. Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjögren's syndrome: proposed criteria for classification. Arthritis Rheum 1986;29:577–85.
2. Chan EK, Hamel JC, Buyon JP, Tan ET. Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen. J Clin Invest 1991;87:68–76.
3. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, et al. Identification of α-fodrin as a candidate autoantigen in primary Sjögren's syndrome. Science 1997;276:604–7.
4. Yanagi K, Ishimaru N, Haneji N, Saegusa K, Saito I, Hayashi Y. Anti-120-kDa α-fodrin immune response with Th-1-cytokine profile in the NOD mouse model of Sjögren's syndrome. Eur J Immunol 1998;28:3336–45.
5. Dighiero G, Rose NR. Critical self-epitopes are key to the understanding of self-tolerance and autoimmunity. Immunol Today 1999;20:423–6.
6. Ridgway WM, Fasso M, Fathman CG. A new look at MHC and autoimmune disease. Science 1999;284:749–51.
7. Harrington CJ, Paez A, Hunkapiller T, Mannikko V, Brabb T, Ahearn M, et al. Differential tolerance is induced in T cells recognizing distinct epitopes of myelin basic protein. Immunity 1998;8:571–80.
8. Hoglund P, Mintern J, Waltzinger C, Heath W, Benoist C, Mathis D. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med 1999;189:331–9.
9. Kojima A, Tanaka-Kojima Y, Sakakura T, Nishizuka Y. Spontaneous development of autoimmune thyroiditis in neonatally thymectomized mice. Lab Invest 1976;34:550–7.
10. Bonomo A, Kehn PJ, Payer E, Rizzo L, Cheever AW, Shevach M. Pathogenesis of post-thymectomy autoimmunity. J Immunol 1995; 154:6602–11.
11. Hayashi Y, Kojima A, Hata M, Hirokawa K. A new mutation involving the sublingual gland in NFS/N mice. Am J Pathol 1988;132:187–91.
12. Haneji N, Hamano H, Yanagi K, Hayashi Y. A new animal model for primary Sjögren's syndrome in NFS/*sld* mutant mice. J Immunol 1994;153:2769–77.
13. Saegusa K, Ishimaru N, Yanagi K, Haneji N, Nishino M, Azuma M, et al. Autoantigen-specific $CD4^+CD28^{low}$ T cell subset prevents autoimmune exocrinopathy in murine Sjögren's syndrome. J Immunol 2000;165:2251–7.
14. Saegusa K, Ishimaru N, Yanagi K, Mishima K, Arakaki R, Suda T, et al. Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjögren's syndrome. J Immunol 2002;169:1050–7.
15. Perrin D, Langley OK, Aunis D. Anti-alpha-fodrin inhibits secretion from permeabilized chromaffin cells. Nature 1987;326: 498–501.
16. Perrin D, Möller K, Hanke K, Söling HD. cAMP and Ca^{2+}-mediated secretion in parotid acinar cells is associated with reversible changes in the organization of the cytoskeleton. J Cell Biol 1992;116:127–34.
17. White SC, Casarett GW. Induction of experimental autoallergic sialadenitis. J Immunol 1974;112:178–85.
18. Moon RT, McMahon AP. Generation of diversity in nonerythroid spectrins: multiple popypeptides are predicted by sequence analysis of cDNAs encompassing the coding region of human nonerythroid spectrin. J Biol Chem 1990;265:4427–33.
19. Brocke S, Gijbels K, Allegretta M, Ferber I, Piercy C, Blankenstein T, et al. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. Nature 1996;379:343–6.
20. Quaratino S, Feldmann M, Dayan CM, Acuto O, Londei M. Human self-reactive T cell clones expressing identical T cell receptor β chains differ in their ability to recognize a cryptic self-epitope. J Exp Med 1996;183:349–58.
21. Saito I, Haruta K, Shimuta M, Inoue H, Sakurai H, Yamada K, et al. Fas ligand-mediated exocrinopathy resembling Sjögren's syndrome in mice transgenic for IL-10. J Immunol 1999;162:2488–92.
22. Delporte BC, Delporte C, O'Connell BC, He X, Lancaster HE, O'Connell AC, et al. Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc Natl Acad Sci U S A 1997;94:3268–73.
23. Von Boehmer H. Developmental biology of T cells in T cell-receptor transgenic mice. Annu Rev Immunol 1990;8:531–56.
24. Miller JF, Kurts C, Allison J, Kosaka H, Carbone F, Heath WR. Induction of peripheral $CD8^+$-T-cell tolerance by cross-presentation of self antigens. Immunol Rev 1998;165:267–77.
25. Herrath MG, Holz A, Homann D, Oldstone MBA. Role of viruses in type I diabetes. Semin Immunol 1998;10:87–100.
26. Singh B, Prange S, Jevnikar AM. Protective and destructive effects of microbial infection in insulin-dependent diabetes mellitus. Semin Immunol 1998;10:79–86.
27. Wong FS, Janeway CAJ. The role of CD4 and CD8 T cells in type I diabetes in the NOD mouse. Res Immunol 1997;148:327–32.
28. Kay TW, Chaplin HL, Parker JL, Stephens LA, Thomas HE. $CD4^+$ and $CD8^+$ T lymphocytes: clarification of their pathogenic roles in diabetes in the NOD mouse. Res Immunol 1997;148: 320–7.
29. Roep BO. T-cell responses to autoantigens in IDDM: the search for the Holy Grail. Diabetes 1996;45:1147–56.
30. Wegmann DR. The immune response to islets in experimental diabetes and insulin-dependent diabetes mellitus. Curr Opin Immunol 1996;8:860–4.
31. Haqqi TM, Anderson GD, Banerjee S, David CS. Restricted heterogeneity in T-cell antigen receptor Vβ gene usage in the lymph nodes and arthritic joints of mice. Proc Natl Acad Sci U S A 1992;89:1253–5.
32. Sumida T, Yonaha F, Maeda T, Tanabe E, Koike T, Tomioka H, et al. T cell receptor repertoire of infiltrating T cells in lips of Sjögren's syndrome patients. J Clin Invest 1992;89:681–5.
33. Zal T, Weiss S, Mellor A, Stockinger B. Expression of a second receptor rescues self-specific T cells from thymic deletion and allows activation of autoreactive effector function. Proc Natl Acad Sci U S A 1996;93:9102–7.
34. Basu D, Horvath S, Matsumoto I, Fremont DH, Allen PM. Molecular basis for recognition of an arthritic peptide and a foreign epitope on distinct MHC molecules by a single TCR. J Immunol 2000;164:5788–96.
35. Wong FS, Karttunen J, Dumont JC, Wen L, Visintin I, Pilip IM, et al. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med 1999;5:1026–31.
36. Zhang J, Markovic PS, Lacet B, Raus J, Weiner HL, Hafler DA. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med 1994;179:973–84.