4-week treatment starting at the age of 4 weeks, and increased both CD4+ and CD8+ T cells after a 2-week treatment starting at the age of 10 weeks. After ED administration of CD4, the resultant CD4+ T cells expressed low levels of CD44 compared with those after treatment with control IgG. The results suggest that the main therapeutic effect of the anti-CD4 mAb in the pathology of SS is the inhbition of CD4+ T cell activation rather than the deletion of CD4+ T cells, although the anti-CD4 mAb both blocks the interaction between CD4 and class II MHC and deletes CD4+ T cells in vivo (27,28). The increase in T cells after ED administration of CD4 would be due to reduced CD4+ T cell activation, resulting in a decrease in the activation-induced death of CD4+ T cells, which may explain the larger relative cell numbers in the anti-CD4 mAb–treated mice than in the control IgG-treated mice. This is supported by results from a previous study (20) demonstrating increased CD4+ T cell apoptosis in humans with SS. Furthermore, in our preliminary experiments, we observed a decrease in annexin V–positive CD4+ T cells in lymph nodes from mice treated with ED administration of CD4 (data not shown).

Nevertheless, it will be important to evaluate the mechanistic basis of the effect of anti-CD4 mAb. In particular, the number of lymphocytes in the lacrimal glands significantly decreased after a 2-week treatment of ED administration of CD4 starting at 10 weeks of age, which suggests the contribution of cytolytic as well as inhibitory activity of anti-CD4 mAb to the inhibition of autoimmune pathology. In addition, the suppressed activation of CD4+ T cells might decrease the activation of antigen-presenting cells or direct the help of CD8+ T cells, which also suppresses the initial expansion of CD8+ T cells, resulting in activation-induced cell death from ages 10 to 12 weeks but not ages 4 to 8 weeks. The differential mode of activation of CD8+ T cells during the initial and progression stages of diseases suggests that CD8+ T cells are required in the progression stage of SS. We are currently performing experiments using anti-CD8 mAb eyedrops to address this issue.

One might argue whether CD25+ regulatory T cells play some role in the effect of ED administration of CD4 in this mouse model because a thymectomy was performed 3 days after birth in the mice with SS. However, we do not think that CD25+ regulatory T cells significantly contribute to the therapeutic effect of ED administration of CD4 because the relative number of CD25+ T cells in regional lymph nodes is not increased in mice treated with ED administration of CD4 (data not shown). Recently, several groups have demonstrated that regulatory T cells differentiated from naive CD25+ T cells (31,32), although there are no appropriate cell surface markers to distinguish such regulatory T cells from effector T cells. Thus, it would be interesting to evaluate the contribution of such regulatory T cells to the inhibition of autoimmune pathology by ED administration of CD4.

ED administration of CD4 inhibited CD4+ T cell activation in cervical lymph node cells but not spleen cells, indicating that anti-CD4 mAb locally affects T cell activation. Although an antibody is a high molecular weight glycoprotein, we have observed the localization of anti-CD4 mAb in the lacrimal glands but not the spleen or regional lymph nodes (Hayashi Y, et al: unpublished observations). Thus, we think that anti-CD4 mAb blocks the activation of pathologic CD4+ T cells by interfering with class II MHC interactions in the lacrimal glands. Nevertheless, it remains unclear how large glycoproteins migrate into the parenchyma of the lacrimal glands, and this must be addressed in a future study.

We showed that ED administration of CD4 reduces serum anti–JS-1 autoantibody levels. These results suggest that ED administration of CD4 inhibited local JS-1–specific CD4+ T cell activation, which impaired T cell help to activate JS-1–specific B cells. Although ED administration of CD4 inhibited autoimmune pathology only in the lacrimal glands, it almost completely eliminated serum JS-1–specific autoantibody. There are 2 possible explanations for this result. The first is that the major helper T cells to induce JS-1–specific autoantibody might be from infiltrating T cells in the lacrimal glands. However, we think this possibility is unlikely because α-fodrin protein is present both in the lacrimal and the salivary glands, and transfer of infiltrating CD4+ T cells into the salivary glands in normal mice is able to cause SS-like lesions with autoantibody production (33). The second possibility is that ED administration of CD4 can prevent T cell help to activate B cells both in the lacrimal and the salivary glands but cannot sufficiently suppress autoimmune pathology in the salivary glands. The direct contribution of CD4+ T cells in the absence of B cells to the autoimmune pathology in this mouse model should be evaluated to address this possibility.

Several groups have examined the effect of ED administration of CSA as a treatment for SS and reported on its positive effect on autoimmune pathology (22,34). However, since systemic injection of CSA induces severe side effects in renal arteries (10), it is possible that even CSA eyedrops can induce some side effects in the eye. In this regard, antibodies have the ability to bind very specifically to their target receptor,

which suggests that it is possible to establish a therapeutic strategy with fewer side effects. Indeed, the application of 2 μg of anti-CD4 mAb daily did not induce any side effects histologically (data not shown). We did not observe any changes in cell number or activation status in the spleen with ED administration of CD4, which repudiates the possibility that anti-CD4 mAb enters into the circulation.

Several aspects of this model system illustrate important issues for future trials with antibody-based therapeutics. A variety of cell surface molecules have been reported to be responsible for the progression of eye symptoms in SS as well as other diseases (17,35,36). Thus, this antibody-based topical therapy may also be applicable to other types of antibodies or diseases.

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Estrogen Deficiency Accelerates Murine Autoimmune Arthritis Associated with Receptor Activator of Nuclear Factor-κB Ligand-Mediated Osteoclastogenesis

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The aims of this study were to evaluate the *in vivo* effects of estrogen deficiency in MRL/*lpr* mice as a model for rheumatoid arthritis and to analyze the possible relationship between immune dysregulation and receptor activator of nuclear factor-κB ligand (RANKL)-mediated osteoclastogenesis. Experimental studies were performed in ovariectomized (Ovx)-MRL/*lpr*, Ovx-MRL+/+, sham-operated-MRL/*lpr*, and sham-operated-MRL+/+ mice. Severe autoimmune arthritis developed in younger Ovx-MRL/*lpr* mice until 24 wk of age, whereas these lesions were entirely recovered by pharmacological levels of estrogen administration. A significant elevation in serum rheumatoid factor, anti-double-stranded DNA, and anti-type II collagen was found in Ovx-MRL/*lpr* mice and recovered in mice that underwent estrogen administration. A high proportion of $CD4^+$ T cells bearing RANKL was found, and an enhanced expression of RANKL mRNA and an impaired osteoprotegerin mRNA was detected in the synovium. An increase in both osteoclast formation and bone resorption pits was found. These results indicate that estrogen deficiency may play a crucial role in acceleration of autoimmune arthritis associated with RANKL-mediated osteoclastogenesis in a murine model for rheumatoid arthritis. (*Endocrinology* 145: 2384–2391, 2004)

IT IS WELL KNOWN that sex steroids have significant impact on the development of autoimmune diseases in both humans and rodents. In particular, estrogen has been suggested to be responsible for the strong female preponderance of the human rheumatoid arthritis (RA), systemic lupus erythematosus, scleroderma, and Sjögren's syndrome (1–3), but the role of estrogens in the female has not been fully characterized. RA is a chronic inflammatory disease characterized by invasive synovial hyperplasia leading to progressive joint destruction. Rheumatoid synovial cells are not only morphologically characterized by their transformed appearance (4) but also are phenotypically transformed to proliferate abnormally (5, 6). These cells invade bone and cartilage by producing an elevated amount of proinflammatory cytokines (7) and matrix metalloproteinases (MMPs) (8) and by inducing osteoclast (OC) formation and activation (9, 10).

OCs, the multinucleated cells exclusively responsible for bone resorption, have been observed to resorb bone actively at the site of invasion of the proliferated synovial membrane into the adjacent bone (11). The cell types responsible for bone resorption in RA have been characterized as authentic OCs (12), and it was reported that rheumatoid synovial fibroblasts are involved in bone destruction by inducing osteoclastogenesis (13). However, the exact mechanisms involved in the formation and activation of OCs in RA are still unclear.

Receptor activator of nuclear facto-κB ligand (RANKL) (14) is a regulator of the immune system and of bone development (15). RANKL is expressed on activated T cells (16), and a major target for RANKL in the immune system appears to be mature dendritic cells (DCs) that express a high level of RANKL receptor (RANK) (17). *In vitro*, RANKL promotes the survival of mature DCs, most likely by up-regulating the expression of Bcl-XL (18), and induces the production of proinflammatory cytokines, such as IL-1 and IL-6, and cytokines that stimulate and induce differentiation of T cells, such as IL-12 and IL-15 (19). Therefore, RANKL is likely to act as a positive-feedback regulator during productive T cell-DC interactions (20).

The MRL/*lpr* mouse strain was chosen to test the estrogenic action because it has a genetic predisposition to arthritis with characteristics similar to those of human RA including cell infiltration, pannus formation, bone and cartilage breakdown, and the presence of serum rheumatoid factor (RF) (21–23). The aim of this study was to analyze the *in vivo* effects of estrogen deficiency on the development of autoimmune arthritis in MRL/*lpr* mice and to evaluate the possible relationship with RANKL-mediated osteoclastogenesis.

Materials and Methods

Mice and treatment

MRL/Mp-*lpr*/*lpr* (MRL/*lpr*, age 4–24 wk; n = 108) and MRL+/+ mice (age 4–24 wk; n = 58) were purchased from Charles River Japan

Abbreviations: Ab, Antibody; CII, type 2 collagen; DC, dendritic cell; dsDNA, double-stranded DNA; ER, estrogen receptor; IFN, interferon; IRF, IFN regulatory factor; LN, lymph node; mAb, monoclonal antibody; MMP, matrix metalloproteinase; OC, osteoclast; OPG, osteoprotegerin; Ovx, ovariectomized; RA, rheumatoid arthritis; RANK, RANKL receptor; RANKL, receptor activator of nuclear factor-κB ligand; RF, rheumatoid factor; sham, sham-operated; TRAP, tartrate-resistant acid phosphatase.

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Inc. (Atsugi, Japan), and were fed under specific pathogen-free conditions. Female MRL/*lpr* mice and MRL+/+ mice (4 wk of age) were ovariectomized (Ovx) and compared with sham-operated (sham)-MRL/*lpr* and MRL+/+ mice. Six to 10 mice in each group were analyzed at 8, 12, 16, 20, and 24 wk of age. Ovx-MRL/*lpr* mice were administered im with 60 mg/kg·wk estrogen (Ovahormine depo; Teikoku Zouki Inc., Tokyo, Japan) in sesame oil or sc with 25 mg/kg·d testosterone (Wako Pure Chemical, Osaka, Japan) from 4–20 wk of age. Care of the mice was in accordance with institutional guidelines.

Histology and immunohistology

All organs were removed from the mice and fixed with 10% phosphate-buffered formalin, and ankles were further decalcified in 10% EDTA. Sections (4 μm in thickness) were stained with hematoxylin and eosin. Histological grading of inflammatory arthritis was done according to the methods by Edwards *et al.* (24) as follows: one point score indicates hyperplasia/hypertrophy of synovial cells; fibrosis/fibroplasia; proliferation of cartilage and bone; destruction of cartilage and bone; and/or mononuclear cell infiltrate. Immunohistological analysis was performed on freshly frozen sections (4 μm in thickness) by the biotin-avidin immunoperoxidase method using ABC reagent (Vector Laboratories Inc., Burlingame, CA). The monoclonal antibodies (mAb) used were biotinylated rat mAbs to CD4, CD8 (BD Biosciences, San Jose, CA), and mouse RANKL (IMGENEX, San Diego, CA).

Flow cytometric analysis

Spleen and inguinal lymph node (LN) cell suspensions were stained with antibodies (Ab) conjugated to phycoerythrin (anti-CD4, Cedarlane Laboratories Ltd., Ontario, Canada; B220, PharMingen, San Diego, CA), fluorescein isothiocyanate (anti-CD8, Cedarlane Laboratories; Thy1.2, PharMingen), and antimouse RANKL (IMGENEX) and analyzed with EPICS (Coulter, Miami, FL).

Measurement of anti-double-stranded DNA (dsDNA) Ab, RF, and type 2 collagen (CII) Ab levels

Anti-dsDNA Abs, RF, and anti-CII Ab were detected by ELISA as described previously (25–27). Briefly, flat-bottom plates (Nalge Nunc International, Roskilde, Denmark) were coated with 1.5 μg/ml of native calf thymus DNA (Life Technologies, Inc., Rockville, MD) in buffer containing 0.1 M sodium bicarbonate and 0.05 M citric acid at 4 C overnight. Serum samples were serially diluted (starting at 1/200) and added to the plates for a 1-h incubation at 37 C. After washing, peroxidase-conjugated goat antimouse IgG, or IgM (Southern Biotechnology Associates, Birmingham, AL), was added and incubated for 1 h at 37 C. Ab binding was visualized using orthophenylenediamine (Sigma, St. Louis, MO). For the measurement of IgG and IgM RF, human IgG and IgM (Chemicon International, Temecula, CA) were coated onto plates at 10 μg/ml in carbonate buffer, and the same procedures were followed as described above. For the measurement of serum Abs to CII, native bovine CII was dissolved in 0.1 M acetic acid at 1 mg/ml and diluted with 0.1 M sodium bicarbonate at 10 μg/ml (pH 9.6). The microtiter plate was coated with 100 μl of CII antigen solution. After washing three times, 100 μl per well of serum samples that had been serially diluted in PBS/Tween 20/1% BSA and control serum were added and incubated for 1 h at 37 C. After washing, peroxidase-conjugated goat antimouse IgG (at 1.4 μg/ml, 100 μl per well) (Organon Teknika, Durham, NC) was added and incubated for 1 h at 37 C. A total of 100 μl o-phenylenediamine (0.5 mg/ml) dissolved in 0.1 M citrate buffer (pH 5.0) containing 0.012% H_2O_2 was added, and the reaction was stopped using 8 N H_2SO_4 (20 μl per well).

Measurement of cytokine production

Cytokine production was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and interferon (IFN)-γ kit (Genzyme, Cambridge, MA). In brief, culture supernatants from LN cells activated with immobilized anti-CD3 mAb (Cedarlane Laboratories) for 3 d were added to microtiter plates precoated with anti-IL-2, IL-4, and IFN-γ capture Ab and incubated overnight at 4 C. After addition of biotinylated detecting Ab and incubation at room temperature for 45 min, avidin-peroxidase was added and incubated at room temperature for 30 min. Finally, 2,2′-azino-di-3-ethylbenzthiazoline sulfonate substrate containing H_2O_2 was added, and the colorimetric reaction was read at an absorbance of 405 nm using an automatic microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The concentrations of IL-2 (picograms per milliliter), IL-4 (picograms per milliliter), and IFN-γ (picograms per milliliter) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

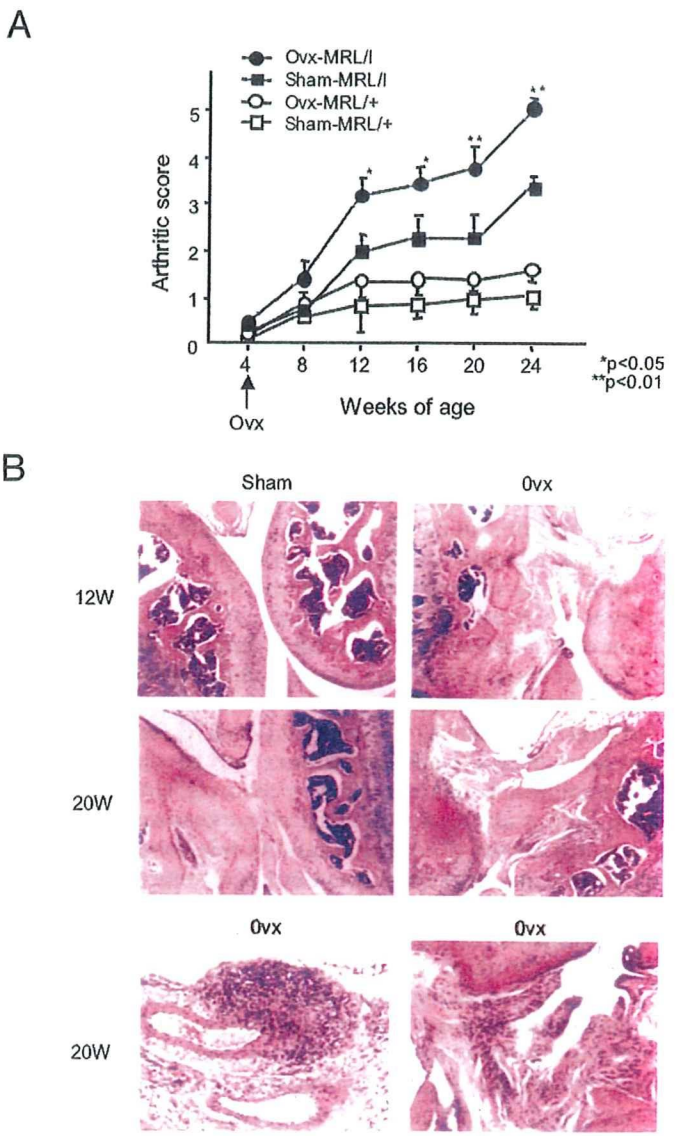

FIG. 1. Effects of the Ovx on joint histopathology. Histological score of autoimmune arthropathy developed in younger Ovx-MRL/*lpr* mice compared with those in sham-MRL/*lpr* and Ovx-MRL+/+ mice until 24 wk of age (A). Histological evaluation of the knee joints was performed according to the methods by Edwards *et al.* (24) (*, $P < 0.05$; and **, $P < 0.01$, Student's *t* test). Representative photomicrographs taken from Ovx- and sham-MRL/*lpr* mice at 12 (12W) and 20 wk (20W) of age (B). The histopathological effects observed in Ovx-MRL/*lpr* mice at age 20 wk included mononuclear cell infiltration into the subsynovial tissue (*lower left*), and synovial hyperplasia (*lower right*) (hematoxylin and eosin). In contrast, mononuclear cell infiltration and bone and cartilage pathology was absent in sham-MRL/*lpr* mice until 20 wk of age (*middle left*).

ELISA for murine osteoprotegerin (OPG)

An anti-murine OPG mAb (TECHNE Corp., Minneapolis, MN) was diluted with 0.1 M sodium bicarbonate (pH 9.6) solution to a concentration of 10 μg/ml, and the 100-μl aliquot was added to each well of 96-well plates. After incubation at 4 C overnight, the capture solution was removed by flicking the plates, and the wells were blocked with the blocking solution (300 μl per well) for 2 h at room temperature. Recombinant OPG (100 μl) (R&D System Inc., Minneapolis, MN) standard and a series of test samples were added to the wells, and the plates were incubated for 2 h at room temperature. The wells were then washed with the washing buffer, and 100 μl of peroxidase-labeled anti-OPG mAb was added to each well. After incubation for an additional 2 h, 100 μl of tetramethylbenzidine substrate reagent was added to each well. Tetramethylbenzidine stop buffer (100 μl) was added to each well, and absorbance at 450 nm of the wells was measured with a microplate reader (Bio-Rad Laboratories Inc.).

RT-PCR

The total RNA from LNs and synovial tissues was extracted as reported previously (28). The RNA was reverse-transcribed into cDNA. The cDNA reaction mixture was diluted with 90 μl of PCR buffer and mixed with 500 nM of the 5′ and 3′ primers, 0.1 mM deoxynucleotide triphosphate mix, 2 mM $MgCl_2$, and 2 U thermostable *Taq* polymerase (PerkinElmer Cetus, Norwalk, CT). The cDNA was subjected to enzymatic amplification in a DNA thermal cycler (PerkinElmer Cetus) by using specific primers. PCR was carried out at 55 C annealing temperature for 30–35 cycles. The specific primers used were as follows: IL-1β, TGA TGA GAA TGA CCT GTT CT and CTT CTT CAA AGA TGA AGG AAA; TNF-α, ATG AGC ACA GAA AGC ATG ATC and AGA TGA TCT GAG TGT GAG GG; IL-6, CTC TGC AAG AGA GAC TTC CAT and ATA GGC AAA TTT CCT GAT TAT A; IFN-γ, CCT CAG ACT CTT TGA ACT CT and CAG CGA CTC CTT TTC CGC TT; IFN regulatory factor (IRF)-1, TCT GAG TGG CAT ATG CAG ATG GAC and GGT CAG AGA CCC AAA CTA TGG TCG; MMP-1, ATG GTG GGG ATG CCC ATT TT and CAG CAT CTA CTT TGT TGC C; MMP-2, GAG TTG GCA GTG CAA TAC CT and GCC ATC CTT CTC AAA GTT GT; MMP-3, GAA ATG CAG AAG TTC CTC GG and GAG TTC CAT AGA GGG ACT GA; MMP-9, CCA TGA GTC CCT GGC AG and AGT ATG TGA TGT TAT GAT G; TIMP (tissue inhibitor of metalloproteinase)-1, CTG GCA TCC TCT TGT TGC TA and AGG GAT CTC CAA GTG CAC AA; RANKL, GGG AAT TAC AAA GTG CAC CAG and GCC ATC CTT CTC AAA GTT GT; RANK, GTC TTC TGG AAC CAT CTT CTC C and CAC AGA CAA ATG CAA ACC TTG; OPG, TCA AGT GCT TGA GGG CAT AC and TGG AGA TCG AAT TCT GCT TG; estrogen receptor (ER)-α, AAT TCT GAC AAT CGA CGC CAG and GTG CTT CAA CAT TCT CCC TCC TC; ER-β, TTC CCA GCA GCA CCG GTA ACC T and TCC CTC TTG GCG CTT GGA CTA; β-actin, GTG GGC CGC TCT AGG CAC CA and CGG TTG GCC TTA GGG TTC AGG GGG. The amplified DNA reaction mixture was subjected to 1.7% agarose gel electrophoresis, and the amplified product was visualized by UV fluorescence after staining with ethidium bromide.

Tartrate-resistant acid phosphatase (TRAP) staining

Staining for TRAP was performed according to the modified method of Takahashi *et al.* (29). Sections were rinsed once with PBS (pH 7.4), air dried, fixed with 10% formalin in PBS (pH 5.4) for 5 min, and fixed with

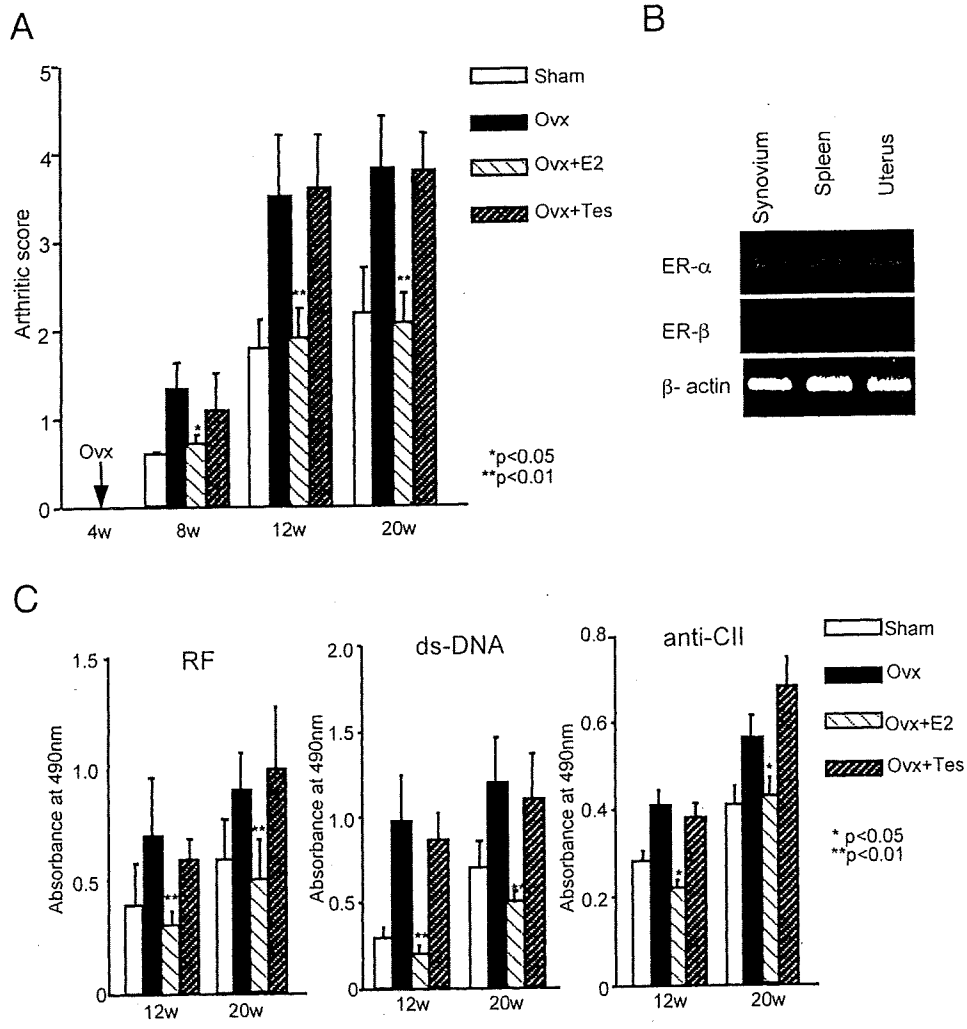

FIG. 2. The destructive lesions in the knee joints in Ovx-MRL/*lpr* mice were inhibited by estrogen administration (10^{-9} M) at ages 12 and 20 wk (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test) (A). Detection of gene expression in ER-α in the synovium but not in ER-β by RT-PCR analysis (B). E2, 17β-Estradiol. Serum RF, anti-dsDNA Abs, and anti-CII were significantly increased in Ovx-MRL/*lpr* mice compared with those in sham-MRL/*lpr* mice, and these changes were entirely recovered by the treatment with estrogen administration (10^{-9} M) at 12 and 20 wk of age (*, $P < 0.05$; and **, $P < 0.01$, Student's *t* test) (C). Tes, Testosterone.

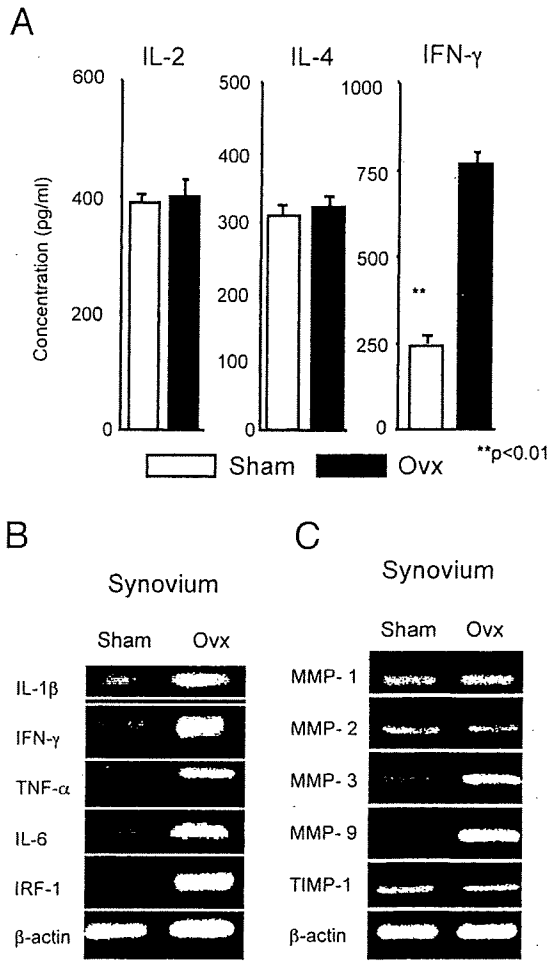

FIG. 3. Effects of the Ovx on expression of cytokines and MMPs. Culture supernatants from anti-CD3 mAb-stimulated LN T cells obtained from Ovx-MRL/*lpr* mice at age 20 wk contained high levels of IFN-γ (**, $P < 0.01$, Student's t test), whereas no different levels of IL-2 and IL-4 was observed by ELISA (A). Increased expressions of cytokine genes including IL-1β, IFN-γ, TNF-α, IL-6, IRF-1, and β-actin were detected in synovium from Ovx-MRL/*lpr* mice at age 20 wk, compared with those from sham-mice by RT-PCR analysis (B). Enhanced gene expressions of MMP-3, and MMP-9 mRNA were found in synovium from Ovx-MRL/*lpr* mice at age 20 wk (C).

methanol-acetone (50:50; pH 5.4) for 30 sec. The coverslips were air-dried and stained for 15 min at room temperature in a 0.1 M sodium acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate and fast red violet LB salt (Sigma) as a stain for the reaction product in the presence of 50 mM of sodium tartrate.

Assessment of bone resorption

Bone marrow cells (5×10^5) from Ovx- and sham-MRL/*lpr* mice were added to the wells of 96-well plates containing a slice of bovine cortical bone and incubated in a total volume of 200 μl α-MEM-fetal bovine serum as described previously (30). All cultures were maintained in the presence of dexamethazone (10^{-7} M, FUNAKOSHI Pharmcol., Tokyo, Japan) and $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M, Chugai Inc., Tokyo, Japan) for 10 d. Bone slices were assessed for bone resorptive activity, brushed with a rubber policeman to remove cells after observation under a microscope, and stained with Mayer's hematoxylin. Bone resorption pits were quantified by densitometric analysis of images of the whole area of bone slices as previously described (30). Additionally, *in vitro* osteoclastogenesis was assayed using bone marrow-derived TRAP-positive cells with macrophage colony stimulating factor (5 ng/ml; PeproTech EC, London, UK), recombinant murine RANKL (10 ng/ml; PeproTech Inc., Rocky Hill, NJ), recombinant OPG (100 ng/ml; R&D Systems Inc.), and 17β-estradiol (10^{-9} M), at indicated concentration after the estimation of dose responses.

Results

Effects of the Ovx on joint histopathology

Destructive autoimmune arthritis developed in young Ovx-MRL/*lpr* mice, not in young sham-MRL/*lpr* and Ovx-MRL+/+ mice, and these lesions aggravated with age from 12 until 24 wk of age. Histological analysis of the knee joints was performed at 8, 12, 16, 20, and 24 wk of age for all the experiments. Analysis of the histological results for the experiment, shown in Fig. 1A, indicates that the group that was treated with Ovx had significant higher subsynovial inflammation, synovial hyperplasia, pannus formation and cartilage erosion, bone destruction, and overall histopathology. Shown in Fig. 1B are photomicrographs taken from representative Ovx- and sham-MRL/*lpr* mice at 12 and 20 wk of age. The effects observed in Ovx-MRL/*lpr* mice included synovial hyperplasia, pannus formation, bone erosion, and infiltration of mononuclear cells into the subsynovial tissue. In contrast, mononuclear cell infiltration and bone and cartilage pathology was absent in sham-MRL/*lpr* mice until age 20 wk.

Recovery of autoimmune arthritis by estrogen administration

The destructive lesions in the knee joints in Ovx-MRL/*lpr* mice were inhibited by estrogen administration (10^{-9} M) at 12 and 20 wk of age (Fig. 2A). Testosterone administration in Ovx-MRL/*lpr* mice resulted in severe inflammatory lesions as the same levels. We confirmed gene expression in ER-α but not ER-β in synovial tissues by RT-PCR analysis (Fig. 2B), indicating that estrogenic action to the synovial tissues might be directly affected through estrogen/ER-α binding *in vivo*. We detected increased levels of serum RF, anti-dsDNA, and anti-CII Abs in Ovx-MRL/*lpr* mice compared with those in sham mice, and these levels were entirely recovered in Ovx-MRL/*lpr* mice that underwent estrogen administration (Fig. 2C).

Effects of the Ovx on cytokine and MMP expression

Culture supernatants from anti-CD3 mAb-stimulated LN T cells obtained from Ovx-MRL/*lpr* mice at 20 wk of age contained high levels of IFN-γ, whereas no difference in levels of IL-2 and IL-4 was observed by ELISA (Fig. 3A). We next analyzed the effects of the Ovx on various gene expressions in the synovial tissues. Increased expressions of cytokine genes including IL-1β, IFN-γ, TNF-α, IL-6, IRF-1, and β-actin mRNA were detected in synovial tissues from Ovx-MRL/*lpr* mice at 20 wk of age, compared with those from sham-MRL/*lpr* mice by RT-PCR analysis (Fig. 3B). In addition, we found elevated gene expressions of MMP-3 and MMP-9 in synovial tissues from Ovx-MRL/*lpr* mice (Fig. 3C). These data suggest that estrogen deficiency induces various gene expressions directly responsible for tissue damage on the development of autoimmune arthritis.

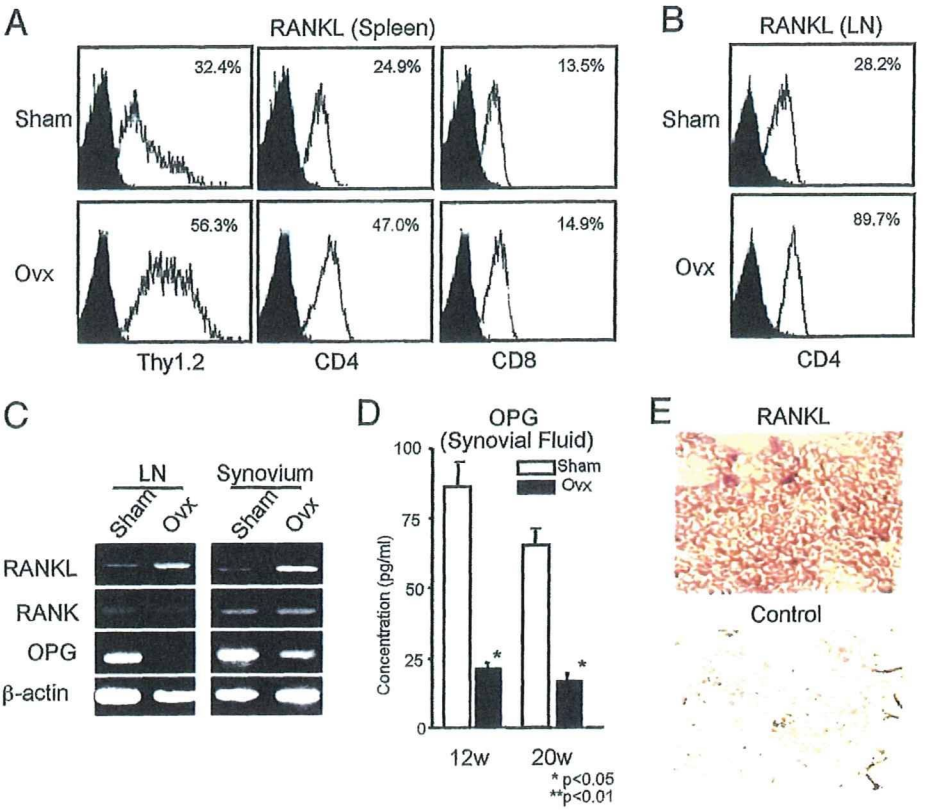

FIG. 4. A significant increase in splenic Thy1.2$^+$ and CD4$^+$ T cells bearing RANKL from Ovx-MRL/lpr mice at 20 wk of age was observed as compared with those from sham-MRL/*lpr* mice, whereas no remarkable change in CD8$^+$ T cells bearing RANKL was found (A). A large proportion of CD4$^+$ T cells in LN bearing RANKL (89.7%) from Ovx-MRL/*lpr* mice was detected on flow cytometry, as compared with that of sham-MRL/*lpr* mice (28.2%) (B). A prominently enhanced RANKL mRNA and an impaired OPG mRNA were observed in LN and synovium from Ovx-MRL/*lpr* mice by RT-PCR analysis (C). A significantly decreased OPG concentration was found in synovial fluid of Ovx-MRL/*lpr* mice at ages 12 (12w) and 20 wk (20w) (*, $P < 0.05$; **, $P < 0.01$, Student's *t* test) (D). A large proportion of infiltrating cells in synovium was positive for RANKL in Ovx-MRL/*lpr* mice at 20 wk of age. Isotype-matched controls were all negative (E).

Effects of the Ovx on RANKL, RANK, and OPG expression

We analyzed the spleen and LN cells bearing RANKL by flow cytometry. A significant increase of Thy1.2$^+$, and CD4$^+$ T cells bearing RANKL in the spleen from Ovx-MRL/*lpr* mice was observed, compared with those from sham-MRL/*lpr* (Fig. 4A). We detected a large proportion of CD4$^+$ T cells in LN bearing RANKL (89.7%) from Ovx-MRL/*lpr* mice as compared with those from sham-MRL/*lpr* mice (28.2%) (Fig. 4B). In addition, an enhanced RANKL mRNA and an impaired OPG mRNA were observed in LN and synovium from Ovx-MRL/*lpr* mice, compared with those in sham-mice by RT-PCR analysis (Fig. 4C). Indeed, a significant decrease in OPG concentration was found in synovial fluid of Ovx-MRL/*lpr* mice compared with those of sham-mice at 12 and 20 wk of age (Fig. 4D). A large proportion of infiltrating cells in synovium was positive for RANKL in Ovx-MRL/*lpr* mice (Fig. 4E).

Effects of the Ovx on OC formation and bone resorption

As seen in Fig. 5A, numerous multinucleated (more than three nuclei), TRAP-positive OC-like cells were detected in the knee joints from both Ovx- and sham-MRL/*lpr* mice. A significant increase in number of OCs formed in the joints from Ovx-MRL/*lpr* mice was observed compared with those from sham-MRL/*lpr* mice (Fig. 5B). We next examined *in vitro* whether the TRAP-positive multinucleated cells (more than three nuclei) from bone marrow cells resorbed bovine bone slices (30). When the bone marrow cells were cultured with dexamethasone and 1α,25-$(OH)_2D_3$, they differentiated into TRAP-positive multinucleated cells, and numerous resorption pits were formed on their surfaces (Fig. 5C). A significantly large number of resorption pits were formed using the bone marrow cells from Ovx-MRL/*lpr* mice compared with those from sham-MRL/*lpr* mice (Fig. 5D).

Discussion

RA is characterized by progressive joint damage that is mediated by several mechanisms (31). Although the etiology of RA remains unknown, joint damage results from the degradation of connective tissue by MMPs and the stimulation of osteoclastogenesis by activated CD4$^+$ T cells (10). Se· hormones influence both humeral and cell-mediated immune response, and estrogen is one of potential factors in this immunological dimorphism (32–34). We have examined the effects of the Ovx on the development of autoimmune arthritis in animals that are susceptible to the development of human RA-like disease. Histology of autoimmune arthritis in Ovx-MRL/*lpr* mice showed severe destructive changes in the younger age examined. It is generally accepted that a severe inflammatory arthritis and systemic autoimmune disease, including glomerulonephritis and autoantibody production, develop in aged (>6-month-old) MRL/*lpr* mice (21, 22). We show here that estrogen deficiency caused a severe inflammatory arthritis in younger (<3-month-old) MRL/*lpr* mice, and these lesions were dramatically prevented by exogenous estrogen treatment. Although the mechanism in detail of estrogen activity still remains unclear, these findings suggest that estrogenic action has an important role during development of autoimmune arthritis in MRL/*lpr* mice. An estrogen deficiency by Ovx in murine RA model results in a significant increase of serum RF, anti-dsDNA Abs, and anti-CII Ab, and these changes were recovered by estrogen ad-

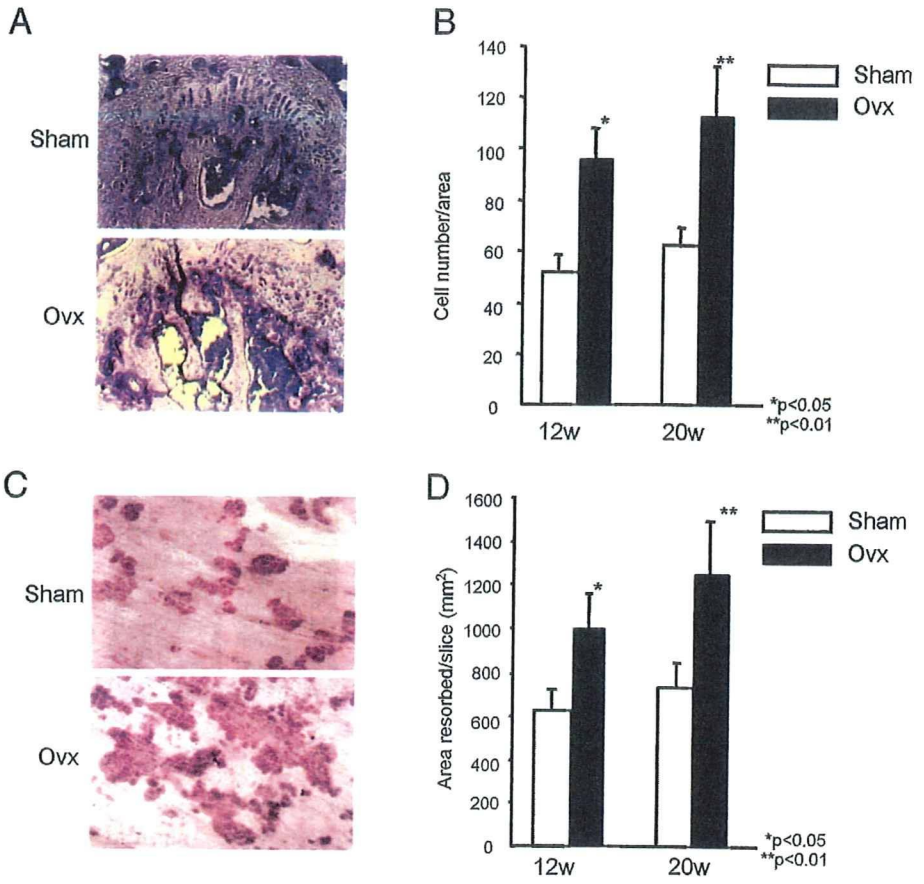

FIG. 5. Effect of the Ovx on OC formation and bone resorption assay. TRAP-positive OC-like cells were detected in the knee joints from both Ovx- and sham-MRL/*lpr* mice (A). A significantly increased number of OCs formed in the joints from Ovx-MRL/*lpr* mice at ages 12 (12w) and 20 wk (20w) was observed compared with those from sham-MRL/*lpr* mice (B) (*, $P < 0.05$ and **, $P < 0.01$, Student's t test). TRAP-positive multinucleated cells (more than three nuclei) from bone marrow cells were observed in both Ovx- and sham-MRL/*lpr* mice when cultured with dexamethasone (10^{-7} M) and $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) (C). A significantly large number of resorption pits was formed using the bone marrow cells from Ovx-MRL/*lpr* mice compared with those from sham-MRL/*lpr* mice (*, $P < 0.05$ and **, $P < 0.01$, Student's t test)(D).

ministration. A previous report has shown that treatment with low doses of β-estradiol exerts a suppressive effect on both development of collagen arthritis as well as T cell-dependent immune reactivity toward type II collagen (35). It was also reported that an estrogen deficiency stimulates B cell development (36) and autoantibody production (36–38), and its increase by an estrogen deficiency has been mediated by cytokines such as IL-6, IFN-γ, and TNF-α (39–41). A recent report has demonstrated that estrogen deficiency induces bone loss by increasing T cell proliferation through IFN-γ-induced class II transactivator (42).

Bone resorption is regulated by the immune system, in which T cell expression of RANKL, which is essential for osteoclastogenesis, may contribute to pathological conditions, such as RA (43). However, it remains unclear whether activated T cells maintain bone homeostasis by counterbalancing the action of RANKL. In this study, a significant increase of $CD4^+$ T cells bearing RANKL in the spleen and inguinal LN from Ovx-MRL/*lpr* mice was observed. Moreover, we detected an enhanced RANKL mRNA expression and $RANKL^+$ infiltrating cells in synovium from Ovx-MRL/*lpr* mice. In contrast, an impaired OPG concentration was found in synovial fluid, in addition to a decreased OPG mRNA in synovium of Ovx-MRL/*lpr* mice. Although molecular mechanisms demonstrating that the appearance of increased RANKL expression in T cells and synovium is related to the development of the autoimmune response are obscure, a role for RANKL in bone resorption in RA is suggested by the identification of RANKL mRNA and protein in cultured synovial fibroblasts from patients with RA and in $CD4^+$ and $CD8^+$ T cells in RA synovial tissues (10). Additional evidence that RANKL plays a critical role in the pathogenesis of bone destruction in inflammatory arthritis comes from studies in the rat adjuvant arthritis model (44, 45). Moreover, it has been recently demonstrated that up-regulation of RANKL on bone marrow cells is an important determinant of increased bone resorption induced by estrogen deficiency (46). Treatment with OPG also prevented OC accumulation, whereas destruction of bone in untreated arthritic animals was accompanied by the accumulation of large numbers of $TRAP^+$ OC-like cells (47). OPG treatment in an animal model of arthritis that is dependent on T cell activation has the potential for blocking not only the effects of RANKL on OC differentiation and activation but also the influence of RANKL on T cell-DC interactions (20).

OCs have a crucial role in the local bone destruction that occurs in association with chronic inflammatory diseases (48). Diseases such as RA have been associated with the accumulation of TNF-α and/or other proinflammatory cytokines such as IL-1 and IL-6, which likely mediate local bone destruction by stimulating OC activity (49). The results in the present study demonstrated an increased gene expression of cytokines including IL-1β, TNF-α, IL-6, IFN-γ, β-actin, and IRF-1 in the synovium from Ovx-MRL/*lpr* mice. In addition, an elevated gene expressions of MMP-3 and MMP-9 mRNA were observed in the synovium. Moreover, we found a significant increase in number of OCs, and bone resorption pits formed in Ovx-MRL/*lpr* mice. It has been shown that estro-

gen prevents bone loss through multiple effects on bone marrow and bone cells, which result in decreased OC formation (50), increased OC apoptosis (51), and decreased capacity of mature OCs to resorb bone (52). Because it was also demonstrated by direct evidence that treatment with estrogens suppressed RANKL-mediated OC formation (53), it is possible that their contribution to the increased osteoclastogenesis and the bone loss has been induced by estrogen deficiency.

In conclusion, we have demonstrated that activation of $CD4^+$ T cells bearing RANKL induced by an estrogen deficiency may play an important role on acceleration of autoimmune arthritis, and estrogenic action appears to influence joint destruction associated with RANKL-mediated osteoclastogenesis in a murine model for RA.

Acknowledgments

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Apoptosis and estrogen deficiency in primary Sjögren syndrome

Yoshio Hayashi, Rieko Arakaki and Naozumi Ishimaru

Purpose of review

Primary Sjögren syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La. The purpose of this review is to discuss recent advances in the pathogenesis of primary Sjögren syndrome.

Recent findings

Although several candidate autoantigens including α-fodrin have been reported in Sjögren syndrome, the pathogenic roles of the autoantigens in initiation and progression of SS are still unclear. It is possible that individual T cells activated by an appropriate self antigen can proliferate and form a restricted clone. Recent evidence suggests that the apoptotic pathway plays a central role in tolerizing T cells to tissue-specific self antigen, and may drive the autoimmune phenomenon. Cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune response. The studies reviewed imply that Fas-mediated cytotoxicity and caspase-mediated α-fodrin proteolysis are involved in the progression of tissue destruction in Sjögren syndrome. Fas ligand (FasL), and its receptor Fas are essential in the homeostasis of the peripheral immune system. It can be considered that a defect in activation-induced cell death of effector T cells may result in the development of autoimmune exocrinopathy in Sjögren syndrome.

Summary

Although the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear, it is possible that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens.

Keywords

Sjögren syndrome, apoptosis, activation-induced cell death, estrogen deficiency

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Abbreviations

AICD	activation-induced cell death
FasL	Fas ligand
SS	Sjögren syndrome
TCR	T-cell antigen receptor

1040–8711

Introduction

Organ-specific autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance [1,2]. Sjögren syndrome (SS) is a T-cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La [3,4]. Autoreactive T cells bearing CD4 molecule may recognize unknown autoantigen triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) [5]. It is now evident that the interaction of Fas with FasL regulates a large number of pathophysiological processes of apoptosis including autoimmune diseases [6]. Recent studies have now confirmed the observation that apoptotic cells in various cell types are implicated as the source of autoantigen when stimulated with different proapoptotic stimuli [7,8]. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases [9], accumulated evidences suggest an important role of apoptosis in disease pathogenesis of Sjögren syndrome [10].

Apoptotic cells in Sjögren syndrome

Recent studies have suggested that the Fas-Fas ligand (FasL) system plays a major role on the induction of apoptosis in target organs with autoimmune diseases such as autoimmune gastritis, Hashimoto thyroiditis, and rheumatoid arthritis [11,12]. It has been reported that both Fas and FasL are present in thyrocytes, and their concomitant expression on thyrocytes, independent of infiltrating T cells, is responsible for thyrocyte destruction in Hashimoto thyroiditis [13]. In contrast, expression of Fas by pancreatic β cells has been shown to have a major influence on the susceptibility of tissue destruc-

tion in nonobese diabetic (NOD) mice to diabetes [14,15•]. Since it was reported that Fas expression was observed in the salivary gland cells with human Sjögren syndrome [16], it was likely that Fas-mediated apoptosis may contribute to tissue destruction in the salivary glands with Sjögren syndrome. A cleavage product of 120-kDa α-fodrin was identified as an important autoantigen in human Sjögren syndrome besides NFS/sld murine model for Sjögren syndrome [17]. α-Fodrin is a ubiquitous, calmodulin-binding protein [18] found to be cleaved by calcium-activated protease (calpain) in apoptotic T cells, and by calpain or caspase 3 [19] in anti-Fas-stimulated Jurkat cells or neuronal apoptosis [20]. It was demonstrated that the fodrin α subunit is cleaved in association with apoptosis, and the 120-kDa fragment is a breakdown product of the mature form of 240-kDa fodrin-α subunit [20,21]. Previous studies have demonstrated evidence that caspase 3 is required for α-fodrin cleavage during apoptosis [21]. In Jurkat cells, caspase 3-like proteases have been reported to cleave α-fodrin and poly (ADP-ribose) polymerase [21]. The observation that ubiquitously expressed autoantigens (e.g., α-fodrin, La, and nuclear mitotic apparatus protein) in Sjögren syndrome are specifically cleaved by granzyme B strongly suggests that a common biochemical event (novel autoantigen cleavage during granule-induced epithelial cell death) is responsible for selecting the unconnected group of molecules [22].

In vitro studies demonstrated that apoptotic mouse salivary gland (MSG) cells result in a specific α-fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked α-fodrin cleavage [23]. A significant increase of TUNEL$^+$-apoptotic epithelial duct cells in the salivary glands was detected in NFS/sld Sjögren syndrome mouse model. MSG cells constitutively express Fas with high proportion, and tissue-infiltrating CD4$^+$ T cells isolated from the salivary gland tissues bear a large proportion of FasL. Importantly, the tissue-infiltrating CD4$^+$ T cells, but not CD8$^+$ T cells, are responsible for tissue destruction as judged by *in vitro* ^{51}Cr release cytotoxic assay against MSG cells *in vitro*. Although it has been reported that Fas-induced apoptosis seems to be the major killing pathway of the CD4$^+$ cytotoxic T cells [24], one mechanism by which activated CD4$^+$ T cells induce cytotoxicity towards salivary gland cells in Sjögren syndrome is Fas based. *In vivo* treatment with caspase-inhibitors, z-VAD-fmk and DEVD-CHO, into murine model results in dramatically inhibitory effects on the development of autoimmune lesions, and in restoration of sicca syndrome [23]. There is increasing evidence that the cascade of caspases is a critical component of the cell death pathway [25,26], and a few proteins have been found to be cleaved during apoptosis. These include poly (ADP-ribose) polymerase, a small U1 nuclear ribonucleoprotein, and α-fodrin, which were subsequently identified as substrates for caspases [27,28]. The development of autoimmune exocrinopathy in Sjögren syndrome appears to be dependent on autoantigen cleavage through caspase cascade, and caspase-inhibitors might provide a new therapeutic option directed at reducing tissue damage.

T cell apoptosis in Sjögren syndrome

Activation-induced cell death (AICD) is a well-known mechanism of peripheral T-cell tolerance that depends upon an interaction between Fas and FasL [29]. AICD plays a central role, especially in killing autoreactive T cells and in preventing autoimmune responses [30]. It has been reported that activation of T-cell clones induces FasL expression, and AICD in autoreactive T cells *in vivo* has been proposed to limit the expansion of an immune response by eliminating effector cells [31••]. Although it can be considered that a defect in AICD of effector T cells may result in the development of autoimmune disease [32], an *in vivo* role of organ-specific autoantigen for AICD is entirely unclear. Because the administration of a soluble form of anti-FasL antibody (FLIM58) results in severe destructive autoimmune exocrinopathy in a murine model of Sjögren syndrome [33], it is possible that an organ-specific autoantigen may play an important role on down-regulation of AICD. A high titer of serum autoantibodies against 120-kD α-fodrin autoantigen was detected in the FLIM58-treated mice, and splenic T-cell culture supernatants contained high level of interferon-γ. FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from the murine Sjögren syndrome model, but not from Fas-deficient MRL/lpr mice and FasL-deficient MRL/gld mice. FasL undergo matrix metalloproteinase–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble trimeric ligands (soluble FasL [sFasL]). In this case, the processing of sFasL occurs in autoantigen-specific CD4$^+$ T cells, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from Sjögren syndrome mouse model [33]. The increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector CD4$^+$ T cells in the murine SS model. Previous studies have demonstrated that CD4$^+$ T cells are susceptible to AICD induced through T-cell receptor–mediated recognition of allogeneic MHC class II molecules, supporting the notion that AICD can be triggered in activated T cells through the T-cell receptor–mediated recognition of antigen [34,35]. Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders [36]. In proteoglycan-induced arthritis, CD4$^+$ T cells proliferate at a high rate in response to proteoglycan stimulation, and exhibit a Th1-type response [37]. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of Sjögren syndrome.

Estrogen deficiency in Sjögren syndrome

Sex hormones influence both humoral and cell-mediated immune response, and estrogen is one of the potential factors in this immunologic dimorphism [38,39]. Estrogenic action has been suggested to be responsible for the strong female preponderance of autoimmune diseases including systemic lupus erythematosus and SS [40,41]. Although a number of autoimmune diseases are known to develop in postmenopausal women, the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear. Previous reports indicate that the increase in autoantibody production as a result of estrogen deficiency is mediated by cytokines such as interleukin-6, interferon-γ (interferon-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 and activation of human monocytes [42–44]. Estrogen deficiency induced by ovariectomy accelerates destructive autoimmune lesions, and these lesions were recovered by estrogen administration in an SS mouse model [45]. It was demonstrated that the dysfunction of regulatory T cells caused by estrogen deficiency may play a crucial role in acceleration of organ-specific autoimmune lesions, and that estrogenic action influences target epithelial cells through Fas-mediated apoptosis [45]. It was also demonstrated that interferon-γ-induced Fas expression on these cells was reduced by the addition of estrogens. Previous studies have shown that physiologic concentration of estrogens augmented the activity of the interferon-γ promotor in mitogen-stimulated murine spleen cells [46], and the administration of exogenous estrogens could induce Fas-mediated apoptosis not only in cultured cells but also *in vivo* [47]. 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 [48,49].

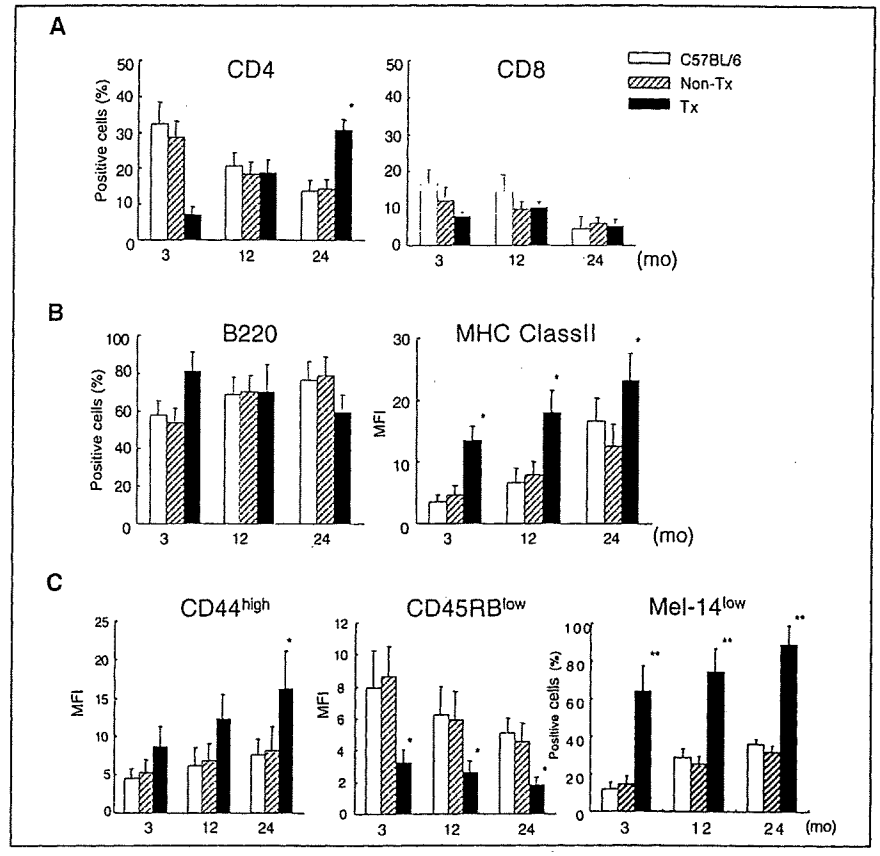
Previous studies concerning gender differences in autoimmunity have suggested that estrogen influences the cytokine production of effector cells and autoantibody production [50,51]. The distinct immune environments in males and females underlie many of the gender-related differences in autoimmunity. 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 [52]. Estrogen withdrawal after menopause leads to an increase in the production of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1, interleukin-6, and tumor necrosis factor-α [53]. Although many studies have described the effects of estrogen on cytokine production in effector cells, much less is known about the effect of estrogen deficiency in target organs of postmenopausal women. Recent data demonstrated significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen-deficient healthy C56BL/6(B6) mice [54••]. Interestingly, inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α-fodrin-reactive T cells in both ovariectomized B6 and ovariectomized SCID mice. It has been suggested that α-fodrin cleavage triggered by estrogen deficiency plays an important role in the development of autoimmune exocrinopathy in SS. In *in vitro* studies using primary cultured MSG and human salivary gland cells (HSG), a cleavage product of 120-kD α-fodrin was detected in cells that had undergone tamoxifen (Tam)-induced apoptosis, not in other type of cells including MCF-7 [54••]. Because 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. A recent report by Morkuniene *et al.* [55] has shown that 17β-estradiol prevents calcium-induced release of cytochrome c from heart mitochondria. When we analyzed whether cysteine 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, the promoter activity of caspase 1 was significantly increased when HSG cells transfected with the promoter-caspase 1 gene were stimulated with Tam.

Conclusion

A cleavage product of 120-kDa α-fodrin was identified as an important organ-specific autoantigen in human SS. The data discussed in this review are strongly suggestive of essential roles of caspase cascade for α-fodrin autoantigen cleavage leading to tissue destruction in autoimmune exocrinopathy in SS. α-Fodrin cleavage by caspases can potentially lead to cytoskeletal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain [56]. It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles [57], assuming that a cleavage product of 120 kDa α-fodrin could be a novel component of an unknown immunoregulatory networks such as cytolinker proteins [58]. *In vitro* T-cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from murine SS. The processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells *in vivo*, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from mouse model. These data indicate that the increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cell (Fig. 1). Moreover, antiestrogenic actions have a potent effect on the proteolysis of α-fodrin autoantigen through up-regulation of caspase 1 activity. It has been strongly suggested that α-fodrin fragments induced by estrogen deficiency may

Figure 1. An organ-specific autoantigen may play an important role on down-modulation of AICD

A cleavage product of 120-kD α-fodrin in the target cells could be induced by estrogen deficiency during apoptosis through caspase activation, in particular caspase 1. Activation-induced cell death (AICD) results from the interaction between Fas and FasL, and activated T cells expressing both Fas and FasL are usually killed either by themselves or by interacting with each other. FasL undergo matrix metalloproteinase (MMP)–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble FasL (sFasL). FasL-mediated AICD is down-regulated by autoantigen stimulation, indicating that the increased generation of soluble FasL inhibits the normal AICD process, leading to the proliferation of autoreactive $CD4^+$ T cells. A defect in AICD may result in the development of autoimmune diseases.



play an important role in the development of autoimmune lesions in SS. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are being further investigated.

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Molecular Analysis of the Human Autoantibody Response to α-Fodrin in Sjögren's Syndrome Reveals Novel Apoptosis-Induced Specificity

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Lymphocyte infiltration of salivary and lacrimal glands leading to diminished secretion and gland destruction as a result of apoptosis is thought to be pivotal in the pathogenesis of Sjögren's syndrome (SS). The cytoskeletal protein α-fodrin is cleaved during this apoptotic process, and a strong antibody (Ab) response is elicited to a 120-kd fragment of cleaved α-fodrin in the majority of SS patients, but generally not in other diseases in which apoptosis also occurs. Little is known about the anti-α-fodrin autoantibody response on a molecular level. To address this issue, IgG phage display libraries were generated from the bone marrow of two SS donors and a panel of anti-α-fodrin IgGs was isolated by selection on α-fodrin immunoblots. All of the human monoclonal Abs (hmAbs) reacted with a 150-kd fragment and not with the 120-kd fragment or intact α-fodrin, indicating that the epitope recognized became exposed after α-fodrin cleavage. Analysis of a large panel of SS patients (defined by the strict San Diego diagnostic criteria) showed that 25% of SS sera exhibited this 150-kd α-fodrin specificity. The hmAbs stained human cultured salivary acinar cells and the staining was redistributed to surface blebs during apoptosis. They also stained inflamed acinar/ductal epithelial cells in SS salivary tissue biopsies, and only partially co-localized with monoclonal Abs recognizing the full-length α-fodrin. Our study shows that in SS patients, neoepitopes on the 150-kd cleaved product of α-fodrin become exposed to the immune system, frequently eliciting anti-150-kd α-fodrin Abs in addition to the previously reported anti-120-kd Abs. The anti-150-kd α-fodrin hmAbs may serve as valuable reagents for the study of SS pathogenesis and diagnostic analyses of SS salivary gland tissue. ***(Am J Pathol 2004, 165:53–61)***

Sjögren's syndrome (SS) is the second most common autoimmune rheumatic disease, causing ocular and oral dryness and extraglandular manifestations in three to four million people in the United States alone.[1–3] The disease is characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of characteristic autoantibodies. Xerostomia and keratojunctivitis sicca are the common clinical signs, but the San Diego SS diagnostic criteria also require a positive salivary gland biopsy or the presence of autoantibodies to the ribonucleoprotein SS-A/Ro for diagnosis;[4] these requirements are not included in the European Economic Committee diagnostic criteria.[5,6] The typical histopathological findings of SS salivary and lacrimal gland tissues include glandular attrition in acinar and ductal epithelia concomitant with lymphoplasmacytic infiltration consisting of predominantly $CD4^+$ cells, but also $CD8^+$, B cells, and plasma cells. Several immune and inflammatory effector pathways seem to be implicated in the ongoing pathology of SS, but our understanding of the initiation factors and the precise mechanism of epithelial cell damage and dysfunction remains limited.

Recent studies have indicated a 120-kd fragment of α-fodrin as a potential important autoantigen in the pathogenesis of primary SS in both a mouse model and in humans.[7–11] Fodrin is an abundant component of the membrane cytoskeleton of most eukaryotic cells. It is composed of heterodimers of an α (240 kd) and a β (235 kd) subunit that share homologous internal spectrin repeats, but have distinct amino- and carboxyl-terminal regions. The α-fodrin subunit is an actin-binding protein that may be involved in secretion[12–14] and has been shown in apoptotic cells to be cleaved by calpain,

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caspases, and an unidentified protease present in T-cell granule content.[15–18] Indeed, treatment of mice with caspase inhibitors prevents induction of SS.[19] Autoantibodies to the 120-kd cleavage fragment of α-fodrin have been detected in patients with primary and secondary SS but also in a few systemic lupus erythematosus (SLE) patients without SS.[7,9,20–22] Different diagnostic criteria for SS have been used in the various studies and differences in the specificity of 120-kd α-fodrin for SS have been observed, which has rendered the importance of 120-kd α-fodrin as a diagnostic marker controversial.

Here, we have further evaluated the incidence and specificity of anti-α-fodrin Ab response in American SS patients and found a correlation between anti-α-fodrin Ab and SS, but a lower prevalence of anti-120-kd α-fodrin Abs in American *versus* Japanese SS patients. We also found that ~25% of SS sera contained Abs against the 150-kd cleavage fragment of α-fodrin. To examine these Abs at a molecular level, we cloned and characterized a panel of hmAbs from SS patients using phage display technology that specifically recognized the 150-kd α-fodrin neoepitope. The anti-150-kd hmAbs were shown to detect 150-kd α-fodrin in apoptotic acinar and ductal salivary gland cells in cell culture, and in SS salivary gland tissue sections, indicating that the hmAbs may be useful diagnostic reagents in SS pathology.

Materials and Methods

Patients

Sera were obtained from 60 SS patients (42 American and 18 Japanese) who fulfilled the San Diego criteria for the diagnosis of SS;[23] 12 rheumatoid arthritis (RA) patients; 12 SLE patients, diagnosed based on American College of Rheumatology criteria; and 10 healthy individuals. Bone marrow from two Caucasian American patients with secondary SS (designated SS23 and SS30) were obtained for Ab library construction.

Western Blot Analysis

α-Fodrin was purified from mouse brain tissue using the method of Cheney and colleagues[24] yielding >95% purity. Mouse α-fodrin exhibits 94% amino acid sequence identity to human α-fodrin. Coomassie staining of mouse brain α-fodrin separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% Tris-HCl gel (Bio-Rad, Hercules, CA) showed an intense band at 240 kd corresponding to intact α-fodrin, but also weaker bands at 180, 150, 120, 80, 50, and 30 kd that corresponded to cleaved α-fodrin because of low levels of constitutive apoptosis, as previously reported.[14,17,24,25] Mouse brain α-fodrin separated by SDS-PAGE was also electroblotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA), the membrane was blocked with 5% nonfat dry milk (Bio-Rad) in phosphate-buffered saline (PBS), pH 7.0, for 30 minutes, and incubated with serum (diluted 1:1000 in PBS), human recombinant Fabs (1 to 20 μg/ml) or mouse anti-α-fodrin mAb AA6 (Affiniti, Exeter, UK) for 1 hour on a rotator. mAb AA6 predominantly recognizes the 240-kd intact form of α-fodrin, but also the 120- and 150-kd cleaved form of α-fodrin. The membrane was washed three times (10 minutes/wash) in PBS and bound serum Ab was detected with horseradish peroxidase-conjugated goat Fab anti-human IgG (H+L) Ab (Bio-Rad). A patient serum was used as internal control in each blotting experiment to adjust for band intensity variations between gels. The intensity of the bands was scored (1 to 5) based on quantification by densitometry. Bound human recombinant Fabs were detected with horseradish peroxidase-conjugated goat anti-human IgG $F(ab')_2$ Ab and bound mouse mAb detected with horseradish peroxidase-conjugated goat anti-mouse IgG Ab (both Jackson) diluted in blocking solution and incubated for 1 hour at room temperature. After washing for 45 minutes with PBS, membranes were rinsed briefly in MilliQ water, and bound enzyme-labeled Ab was visualized using chemiluminescent substrate (SuperSignal, WestPico; Pierce, Rockford, IL) according to the manufacturer's instructions and autoradiographic film (Eastman Kodak, Rochester, NY). All incubations were done at room temperature. As controls, all experiments were performed using the anti-Ebola virus Fab ELZ510, the anti-HIV-1 gp120 Fab b12, normal sera or by omitting the primary Ab.

Analysis of Patient Sera and Human Fabs by Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse brain α-fodrin (2 μg/ml) and ovalbumin (4 μg/ml) (Pierce) were coated onto microtiter wells (Costar, Cambridge, MA) at 4°C overnight. Wells were washed with PBS; blocked with 4% nonfat dry milk in PBS for 30 minutes; and incubated with patient serum (diluted 1:100 and 1:400 in PBS), human Fabs, or mouse anti-α-fodrin mAb AA6 for 1 hour at 37°C. Wells were washed six times with PBS-0.05% Tween and bound Ab was detected with alkaline phosphatase-conjugated goat IgG anti-human IgG $F(ab')_2$ Ab or anti-mouse IgG $F(ab')_2$ Ab (both 1:500 in 1% bovine serum albumin/PBS, Pierce) and visualized with nitrophenol substrate (NPP substrate) (Sigma, St. Louis, MO) by reading absorbance at 405 nm.

RNA Isolation and Library Construction

RNA was isolated from bone marrow of two American SS patients (designated SS23 and SS30) by a guanidinium isothiocyanate method, as described previously.[26] Serum samples from each donor were drawn concomitantly. After reverse-transcription, the γ1 (Fd region) and κ and λ chains were amplified by polymerase chain reaction and phage-display libraries were constructed in the phage-display vector pComb3, as described previously.[27–29]

Ab Library Selection

Libraries were selected against α-fodrin blotted membrane. Mouse brain α-fodrin was separated by SDS-

PAGE using a 10% Tris-HCl gel and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk/PBS for 30 minutes, the membrane was incubated with phage (10^{11} pfu) resuspended in PBS containing 1% bovine serum albumin for 2 hours at room temperature. The membrane was washed and the bound phage, enriched for those bearing antigen-binding surface Fabs, were eluted with 0.2 mol/L glycine-HCl buffer, pH 2.2, as previously described.[29–31] The eluted phages were amplified by infection of *Escherichia coli* and superinfection with M13 helper phage. The libraries were panned for four consecutive rounds with increasing washing stringency (2 × 10 minutes for first panning round and 4 × 10 minutes thereafter). Phagemid DNA, isolated after the last round of panning, was digested with *Nhe*I and *Spe*I restriction endonucleases and religated to excise the cpIII gene. The reconstructed phagemid was used to transform XL1-Blue cells (Stratagene, La Jolla, CA) to produce clones secreting soluble Fab fragments. Positive Fab clones were purified from bacterial supernatants by affinity chromatography as previously described.[32]

Nucleic Acid Sequencing

Nucleic acid sequencing was performed on a 373A or 377A automated DNA sequencer (ABI, Foster City, CA) using a *Taq* fluorescent dideoxy terminator cycle sequencing kit (ABI). Sequencing primers were as reported.[33] Comparison to reported Ig germline sequences from GenBank and EMBL was performed using the Genetic Computer Group (GCG) sequence analysis program.

Confocal Laser-Scanning Microscopy Analysis of Human Cells and Tissue Biopsies

Human salivary gland (HSG) cells were grown in minimum essential medium, Eagle's, in Earle's balanced salt solution (EMEM) medium supplemented with 10% fetal calf serum and allowed to adhere to chambered coverslips (Nunc, Kamstrup, Denmark) for 48 hours at 37°C, 5% CO_2, to form monolayers. Apoptosis of HSG cells was induced by incubating the cells with 100 ng/ml of tumor necrosis factor-α (Upstate Biotechnology, Lake Placid, NY) and 1 μg/ml of cycloheximide for 3 to 15 hours at 37°C/5% CO_2. Untreated cells or those induced to undergo apoptosis were fixed by 96% ice-cold ethanol for 5 minutes at 4°C or by 4% paraformaldehyde for 10 minutes at room temperature. Paraformaldehyde-fixed cells were washed in PBS before being permeabilized in 0.005% saponin for 10 minutes at room temperature. After washing in PBS and blocking with 5% normal goat serum for 1 hour, cells were incubated with Ab. Fresh-frozen tissue was obtained from labial biopsies of patients with active SS and healthy controls. Freshly cut 5-μm sections were dried overnight, fixed by ice-cold 96% ethanol for 5 minutes at 4°C or by acetone for 10 minutes at room temperature, and blocked with 5% normal goat serum. HSG cells and tissue sections were incubated with human Fabs (10 μg/ml in PBS), or mouse anti-α-fodrin mAb AA6 (Affiniti) for 1 hour. In some experiments apoptotic cells were stained with Annexin V-FITC (Pharmingen, La Jolla, CA) for 1 hour before fixation. The slides were washed with PBS and incubated with fluorescein isothiocyanate-labeled $(Fab')_2$ goat anti-human IgG $(Fab')_2$ Ab (Jackson), and Texas Red-labeled goat anti-mouse IgG Ab (Jackson), or propidium iodine (Sigma) for 1 hour. The slides were again washed with PBS for 5 minutes and mounted with Slow Fade in PBS/glycerol (Molecular Probes, Eugene, OR) before analysis using a Zeiss Axiovert S100 TV confocal laser-scanning microscope (Zeiss, New York, NY). All incubations were performed at room temperature unless otherwise indicated. As controls, all experiments were performed using the human Fab b12 to HIV-1 gp120 or by omitting the primary Ab. Adjacent tissue sections were hematoxylin and eosin stained or stained with anti-CD3 (DAKO, Carpinteria, CA), and anti-cytokeratin 18 (CY-90; Sigma-Aldrich) mAbs to determine the cell type present.

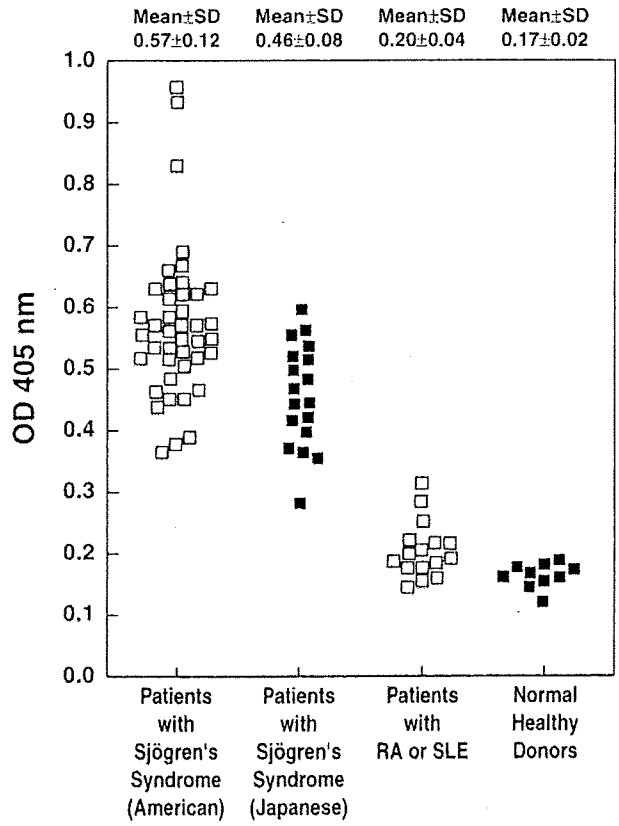

Figure 1. Sera from SS patients contain anti-α-fodrin Abs, as measured by ELISA. Sera, diluted 1:400 in PBS, from 42 American SS patients, 17 Japanese SS patients, 16 RA and SLE patients, and 10 healthy individuals were tested for binding to mouse brain α-fodrin by ELISA. Samples with A_{405} values more than twice the mean of the control normals (>0.33) were considered positive.

Results

Serological Analysis of α-Fodrin Autoantibodies in SS Patients

To investigate the specificity and sensitivity of anti-α-fodrin Abs for SS, serum from patients with SS, RA, SLE,

and healthy individuals were tested for binding to mouse brain α-fodrin by ELISA. A secondary Ab capable of detecting both IgG and IgA was used, because anti-α-fodrin Ab of both the IgG and IgA have been suggested to be elevated in SS sera. As shown in Figure 1, elevated α-fodrin Ab levels were observed in both American and Japanese SS patients compared to the RA and SLE patient groups and healthy individuals. Positivity was defined as an OD_{405} value greater than twice the mean of the normal controls (>0.33) at a serum dilution of 1:400. Sera from all of the American SS patients and all but one of the Japanese SS patients were positive for α-fodrin Abs (98%), whereas only 1 of 16 RA/SLE patients (6%), and none of the healthy donors, were positive. The mean level of anti-α-fodrin Ab in both the American (OD_{405} nm, 0.57 ± 0.12) and Japanese SS patients (OD_{405} nm, 0.46 ± 0.08) were significantly higher than healthy controls (0.17 ± 0.02, $P < 0.0001$) or the RA/SLE patients (0.20 ± 0.04, $P < 0.0001$). The mouse anti-α-fodrin mAb AA6 was included in each experiment as a control (OD_{405} nm, 0.8).

The frequency of SS sera with Abs specific for the 120-kd fragment of cleaved α-fodrin was determined by assessing binding to Western blots of mouse brain α-fodrin. Fifteen of 42 American SS sera (36%) (diluted 1:1000) exhibited reactivity against the 120-kd fragment of α-fodrin (Table 1). In addition, 10 sera showed reactivity with a 150-kd fragment of cleaved α-fodrin (24%), while 7 showed reactivity with a 180-kd fragment and other cleaved products of α-fodrin. As shown in Table 1, some patient sera reacted with multiple α-fodrin fragments, whereas the Western blot for other sera revealed reactivity with only one of the fragments. Overall, 22 American SS sera (52%) reacted with at least one form of cleaved α-fodrin. When the 17 Japanese SS sera were tested by Western blotting, the prevalence of anti-α-fodrin Ab reactivity was found to be significantly higher than in the American SS sera (12 of 17 positive, 70.6%; $P < 0.01$). All of the α-fodrin-reactive sera from the Japanese patients recognized 120-kd, but five also bound to the 150-kd α-fodrin (29.4%) (Table 1). Serum of 8 RA patients, 8 SLE patients, and 10 healthy individuals was also tested by Western blot analysis. Only one SLE serum was found to react with the 120-kd fragments of α-fodrin and none reacted with the 150-kd fragments of α-fodrin.

Table 1. Binding of American (ASSP) and Japanese (JSSP) SS Patient Sera to Cleaved Mouse Brain α-Fodrin by Western Blot Analysis

Patient	Intensity* of bands	Fragment size (kd)
ASSP1	–	
ASSP2	–	
ASSP3	1+	150
ASSP4	–	
ASSP5	–	
ASSP6	1+	120
ASSP7	3+	180
ASSP8	3+	120, 150, 50
ASSP9	2+	120
ASSP10	–	
ASSP11	–	
ASSP12	1+	150, 80, 30
ASSP13	–	
ASSP14	–	
ASSP15	–	
ASSP16	–	
ASSP17	–	
ASSP18	–	
ASSP19	1+	120, 150
ASSP20	1+	120, 150
ASSP21	–	
ASSP22	2+	150, 180
ASSP23	4+	120, 150
ASSP24	–	
ASSP25	2+	150
ASSP26	–	
ASSP27	2+	150
ASSP28	3+	120, 180
ASSP29	2+	120, 180
ASSP30	5+	150
ASSP31	3+	120
ASSP32	–	
ASSP33	–	
ASSP34	3+	120
ASSP35	2+	120, 180, 50
ASSP36	1+	120
ASSP37	–	
ASSP38	–	
ASSP39	1+	120, 180, 50
ASSP40	–	
ASSP41	2+	120, 180, 80
ASSP42	2+	120, 50
JSSP1	3+	120
JSSP2	2+	120
JSSP3	2+	120, 150
JSSP4	2+	120
JSSP5	2+	120, 150
JSSP6	1+	120
JSSP7	1+	120, 150
JSSP8	1+	120
JSSP9	1+	120
JSSP10	1+	120, 150
JSSP11	1+	120
JSSP12	1+	120, 150
JSSP13	–	
JSSP14	–	
JSSP15	–	
JSSP16	–	
JSSP17	–	

*The intensity was evaluated at a scale 1 to 5; 5 being the most intense.

Isolation of Human IgG mAbs Against the 150-kd Form of Cleaved α-Fodrin from SS Patients

To characterize the anti-150-kd α-fodrin Abs response at a molecular level, IgG_1 κ/λ Ab phage display libraries were generated from two patients (SS23 and SS30), whose sera predominantly recognized the 150-kd cleavage fragment of α-fodrin and also reacted with extract of human SS salivary gland tissue. As starting material for the Ab library construction, bone marrow was obtained from patients SS23 and SS30. Bone marrow has been shown to be a major repository for the plasma cells that produce the Abs found in the circulation. The Ab libraries from patients SS23 (≈6 × 10^6 members) and SS30 (≈8 × 10^6 members) were panned against mouse brain α-fodrin consisting of mostly intact α-fodrin, but also a small amount of apoptotic cleaved α-fodrin. The α-fodrin preparation was either separated by SDS-PAGE and

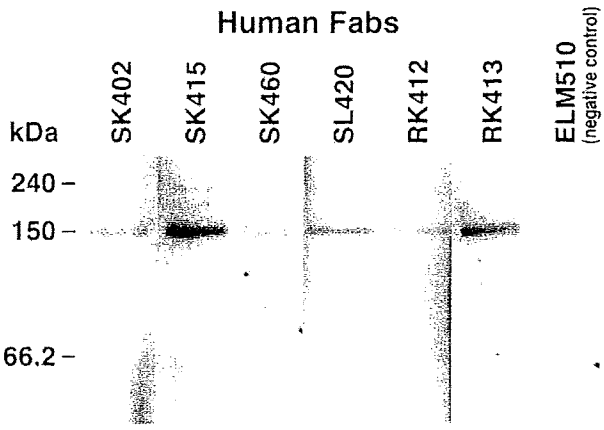

Figure 2. Binding of human monoclonal IgG Fabs to purified mouse α-fodrin brain extract by Western blot analysis. All of the Fabs specifically bound to the 150-kd fragment of cleaved α-fodrin and did not react with 240-kd intact α-fodrin. The human monoclonal anti-Ebola virus Fab ELZ510 was used as a negative control.

transferred to a polyvinylidene difluoride membrane or coated on microtiter wells. After four rounds of biopanning against α-fodrin blotted on a polyvinylidene difluoride membrane, a significant increase in eluted phage was observed, indicating enrichment for antigen-binding Fab-phages. Individual clones, expressed as soluble Fabs by excising the gene III from the pcomb3 phagemid DNA from the last round of selection, were tested for binding to α-fodrin by Western blotting. This analysis yielded 19 Fabs that exhibited strong binding to the 150-kd fragment of cleaved α-fodrin and no binding to intact α-fodrin (240 kd), although present in significant excess, or the 120-kd cleaved form of α-fodrin (Figure 2). The Fabs also failed to react with Western blots of HeLa lysate, which contained intact α-fodrin, but not 120- or 150-kd α-fodrin, as determined by staining with mouse mAb AA6. Sequencing the DNA encoding the heavy chain variable region of the 19 Fabs revealed that 4 Fabs isolated from patient SS23 (SK402, SK415, SK460, and SL420) and 2 Fabs isolated from patient SS30 (RK412 and RK413) were unique, whereas the remaining sequences were repeats of the six sequences (Table 2).

The IgG-Derived Anti-150-kd α-Fodrin Fabs Are Derived as a Result of an Affinity-Matured Antigen-Driven Antibody Response

Next, the variable heavy and light chain genes of the IgG-derived anti-150-kd α-fodrin Fab fragments were compared with the closest germline sequences in the GenBank database (Table 2). The Ab heavy chain is the major contributor to antigen-binding in many instances and so detailed analysis was focused on this chain. All of the variable heavy chain region genes of the anti-150-kd α-fodrin IgG Fabs were highly mutated, and exhibited a high replacement (R) to silent (S) mutation ratio (R/S ratio) for the complementarity determining regions (CDRs) (CDR1 and CDR2) compared to the framework regions (FR) (FR1, FR2, and FR3), characteristic of an affinity-matured antigen-driven Ab response. No particular restriction in the VH or JH gene usage of the anti-150-kd α-fodrin IgG Fabs was observed (Table 2), neither did the analysis reveal any restriction in the germline usage within the context of the VH families used. Unequivocal identification of the closest germline D segment proved impossible because of significant somatic modification.

Epitope Characterization

To pinpoint the epitopes recognized by the anti-150-kd α-fodrin human Fabs, three polypeptides spanning α-fodrin were coated on ELISA wells and tested for reactivity with the Fabs. The polypeptides included JS-1 (1 to 1784 bp), 2.7A (2258 to 4884 bp), and 3'DA (3963 to 7083 bp). None of the tested Fabs recognized any of the peptides, suggesting that the Fabs either recognized regions or partial conformational epitopes of α-fodrin not represented by the peptides.

Subcellular Distribution of 150-kd α-Fodrin in HSG Cells

To determine the subcellular distribution of the 150-kd fragment of cleaved α-fodrin, HSG cells were stained with selected human Fabs and examined by laser-scanning confocal microscopy. As shown in Figure 3, Fab SK415 and SK460 exhibited cytoplasmic staining, whereas no staining was observed with a control Fab. Similar cytoplasmic staining was also observed with mouse mAb AA6 recognizing both intact and cleaved α-fodrin (Figure 3I). As previously reported, the cytoplasmic staining of the mouse mAb AA6 was particularly intense, corresponding to the plasma membrane, but this was not observed with the human anti-150-kd α-fodrin Fabs. The fixation method of the HSG cells (ethanol or paraformaldehyde/saponin) did not seem to influence the staining patterns of Fab SK415 and mAb AA6.

Table 2. Deduced Amino Acid Sequences of the Heavy Chain CDR3 Region and Adjacent Framework Regions of Anti-150 kd, α-Fodrin IgGs

Fab	VH/germline	FR3	CDR3	FR4	JH
SK402 (3)	VH1-8	YYCAR	EGWPPTNDY	WGQ	JH4
SK415 (1)	VH3-21	YFCVR	DLCGGRDS	WGQ	JH5
SK460 (2)	VH3-33	YLCAR	EAWHDIGEYDGRRTLGSVPSLDL	WGQ	JH5
SL420 (1)	VH3-21	YYCAR	DGDGYRDY	WGQ	JH4
RK412 (8)	VH1-69	YYCAR	GFGGEDAYYDNFGYYASTEF	WGL	JH3
RK413 (4)	VH4-4	YYCAR	WGPRDLSGRSGGFDP	WGP	JH4

Number in parentheses denotes the number of Fabs that had the same heavy-chain CDR3 sequence. The closest germline gene, VH and JH families for each clone are also shown.