Table 2
Comparison of deletion frequency between anti-GPI Abs (+) and anti-GPI Abs (−)

Anti-GPI Abs	39 deletions	Deletions/GPI groups		p values
HC(−)				
1	ds:2	2/13	5/78 (6.4%)	$p < 0.001$
2	d:1	1/19		
3	0	0/16		
4	d:1, ds:1	2/15		
5	0	0/15		
HC(+)				
1	d:4, ds:1	5/16	23/73 (31.5%)	
2	ds:1	1/12		
3	d:5, ds:1	6/12		
4	d:3, ds:3	6/20		
5	d:1, ds:4	5/13		
RA(−)				
1	0	0/18	1/63 (1.6%)	$p < 0.0001$
2	ds:1	1/5		
3	0	0/8		
4	0	0/12		
5	0	0/20		
RA(+)				
1	d:1, ds:2	3/14	22/77 (28.6%)	
2	d:2, ds:2	4/10		
3	ds:2, d:2	4/22		
4	d:6, ds:2	8/17		
5	d:1, ds:2	3/14		

p value by χ^2 test. Anti-GPI Ab Pos. vs. Neg. $p < 0.0001$.
d, deletion with no flame shift. ds, deletion with flame shift was found.

autoantibodies against intact GPI are produced through an epitope spreading mechanism, especially in the inflammatory arthritides [25].

Why does this modification occur? Matsumoto et al. [5] reported that soluble GPI protein level decreased with age in a K/BxN arthritis model, and that such decrease triggered T cell perturbation toward GPI [5]. These changes suggested that protein modification can activate autoreactive T cells. Several groups identified the presence of modified GPI proteins in patients with various conditions [17–22], although we did not identify such proteins in our subjects. Deleted protein of myelin proteolipid protein (PLP) in patients with multiple sclerosis is highly expressed in the thymus, and such expression is associated with induction of autoantibody production by autoreactive T cells [26,27]. It is possible that the spontaneous post-transcriptional modification of autoantigens alters the activation of autoreactive T cells, and hence autoantibody production. In fact, we have already identified GPI reactive T cells in the PBMC of patients with GPI variants (Kori et al., submitted).

In conclusion, our results provided evidence that the presence of GPI variants is associated with the production of autoantibodies against GPI. Although the mechanism of post-transcriptional alteration of GPI antigen remains unclear, the GPI variants seem to play a key role in the generation of anti-GPI Abs. Further research using recombinant GPI variant proteins should shed more light on the mechanisms underlying the production of pathogenic antibodies against autoantigens.

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Expression of Tristetraprolin (G0S24) mRNA, a Regulator of Tumor Necrosis Factor-α Production, in Synovial Tissues of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To determine the significance of tristetraprolin (TTP) gene expression in synovial tissues of patients with rheumatoid arthritis (RA).

Methods. Gene expression was examined in synovial tissue and peripheral blood lymphocytes of a patient with RA by differential display-polymerase chain reaction (PCR). One of the identified genes, TTP, was selected for further analysis. cDNA was prepared from synovial tissues of 22 patients with RA and 22 with osteoarthritis (OA). Expression of TTP and tumor necrosis factor-α (TNF-α) genes was measured by TaqMan real-time semiquantification PCR. In RA samples, expression of TTP mRNA was compared with TNF-α mRNA, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and steroid and/or disease modifying antirheumatic drug use.

Results. Expression of TTP gene was significantly higher in synovial tissues of RA patients than in OA. There was no apparent relationship between expression of TTP and TNF-α genes. TTP gene expression had a tendency to be inversely correlated with serum CRP, measured immediately before surgery. In addition, CRP was higher in patients with a low TTP/TNF-α gene expression ratio ($p = 0.0071$, Spearman rank correlation).

Conclusion. A low TTP/TNF-α gene expression ratio could indicate failure of RA patients to produce adequate amounts of TTP in response to increased TNF-α production. Inappropriate TTP production may be one factor that contributes to higher RA disease activity. (J Rheumatol 2004;31:1044–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
DIFFERENTIAL DISPLAY
TRISTETRAPROLIN
TUMOR NECROSIS FACTOR-α

Rheumatoid arthritis (RA) is a chronic disorder of unknown pathogenesis associated with polyarthropathy. Persisting inflammation in the joints may lead to total destruction of joints, causing a great reduction of quality of life of patients with RA. Among a number of cytokines involved in the pathogenesis of arthritis in RA, tumor necrosis factor-α (TNF-α) is one of the most important. TNF-α is produced by macrophages and synovial cells, and induces an array of inflammatory cytokines, chemokines, adhesive molecules, and proteinases. TNF-α is present in synovial fluids of patients with RA[1-3], but not in those of patients with osteoarthritis (OA)[3]. Interestingly, while transgenic mice carrying the complete human TNF-α gene develop normally, transgenic mice carrying a 3' modified human TNF-α transgene have dysregulated TNF-α expression and develop chronic inflammatory arthritis resembling human RA, which could be completely prevented by treatment with anti-TNF-α antibodies[4]. Thus, dysregulated TNF-α production is responsible for arthritis in this animal model, and the 3' region of the TNF-α gene is important for properly controlled production of this cytokine. Subsequent studies and the recent introduction of anti-TNF-α therapies have shown that this cytokine plays a key role in the pathogenesis of human RA, and is a potential target for therapy. However, a better understanding of the disease is needed to develop new methods of therapies, and to prevent total destruction of the joints.

To investigate the molecular mechanisms involved in the pathogenesis of RA, and to search for possible targets for RA-specific therapies, we focused on identifying genes that are specifically expressed in synovial tissues of patients with RA. We used the differential display-polymerase chain reaction (DD-PCR) method to identify known and unknown genes specifically expressed in a given tissue[5,6]. Among a

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number of candidate genes identified, we focused on tristetraprolin (TTP), since TTP is an intracellular protein involved in the degradation of TNF-α mRNA. TTP binds to the 3' untranslated region (3'-UTR) of the TNF-α mRNA, and induces its instability, as reviewed by Blackshear[7]. Thus, TTP is a natural regulator of TNF-α production[8], and theoretically may have a protective role in the inflammatory process of RA. We report that TTP mRNA is highly expressed in RA synovial tissues, compared to its expression in OA synovial tissues. We also examined the relationships between TTP gene expression and TNF-α gene expression, serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF) in patients with RA before surgery.

MATERIALS AND METHODS

Differential display-PCR. Samples from a patient who fulfilled the American College of Rheumatology criteria for classification of RA[9] were used with written informed consent. Total RNA was extracted from synovial tissue from the operated knee joint and Ficoll (Amersham Bioscience Japan, Tokyo. Japan) separated peripheral blood mononuclear cells (PBMC), using Isogen (Nippon Gene, Tokyo. Japan), following the manufacturer's recommended protocol. RNA was reverse transcribed to cDNA using a RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) following the manufucturer's instructions. Gene expression in synovial and PBMC samples was compared by DD-PCR. DD-PCR was carried out using the delta-differential display kit (Clontech. Palo Alto, CA, USA) as described in the manual, with the exception that we visualized the bands on the polyacrylamide gel by silver stain. Bands visible only in amplified products from the synovial sample were excised from the gel, and were cloned into pCRII vectors (Invitrogen Japan, Tokyo, Japan) and sequenced using standard protocols by a sequencer (ABI Prism 310 gene analyzer; Perkin Elmer, Wellesley, MA, USA). A total of 105 genes considered to be specifically expressed by the synovial tissue sample were analyzed in this way. Sequences were searched for homologies by the NCBI BLAST system on the Internet [cited January 20, 2004; available from http://www.ncbi.nih.gov/blast/].

Semiquantitative PCR. Genes identified by DD-PCR were studied in the literature for possible relationships with cell proliferation or survival, inflammation, and immunological functions such as antibody or cytokine production. TTP and several other genes that were considered of possible interest were selected and their expression in synovial tissues from 22 patients with RA and 22 with OA were measured using the TaqMan PCR real-time semiquantification method. All samples were taken with donors' written informed consent, and the study was approved by the local ethical committee. Sera from these patients were obtained 0–2 days before surgery. At the time of serum sampling, patients were taking 0–10 mg/day prednisolone and 0–3 disease modifying antirheumatic drugs (DMARD) including methotrexate (8 mg/week maximum), salazosulfapyridine (1000 mg/day maximum), and 100–200 mg/day bucillamine, a DMARD commonly used in Japan. Total RNA was extracted from synovial tissues from operated knee joints, and cDNA was synthesized using the RevertAid first-strand cDNA synthesis kit. Synthesized cDNA samples were amplified with specific primers and fluorescence-labeled specific probes for the gene of interest, and accumulation of amplified products was monitored with an ABI 7700 sequence detector (Applied Biosystems Japan, Tokyo, Japan). PCR mixture (qPCR Mastermix) was purchased from Eurogentec (Seraing, Belgium); magnesium concentration was 5 mM final, primer concentrations 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were 50°C for 2 min, 95°C for 10 min, then 55 cycles of 95°C for 15 s, and 60°C for 1 min. Standard samples were included and standard curves for the gene of interest and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were generated in every assay. All measurements were done in triplicate. The level of gene expression was calculated from the standard curve, compensated with that of GAPDH gene, and was expressed as a ratio. The sequences of specific primers and probes are as follows: TTP forward: 5'GGCGACTCCCCATCTTCAAT3', TTP probe: 5'TCTGAGTGACAAAGTGACTGCCCGGTC3', TTP reverse: 5'CAGTGCAAGAGACGTGGCTC3'; mortality factor 4 forward: 5'TGCCGAAATTCTTG CAGATT3', mortality factor 4 probe: 5'TCCCGATGCACCCATGTCCC3', mortality factor 4 reverse: 5'AGATGTGGCACTCCATACACC3'; CD63 forward: 5'TTCTTGCTCTACGTCCTCCTG3', CD63 probe: TGGCCTTTTGCGCCTGTGC, CD63 reverse: 5'CACGGCAATCAGTCCCAC3'. Ki autoantigen forward: 5'AAAGCCGCAGACCCTGG3', Ki autoantigen probe: 5'CTCTGGTGGCTAGGGATGTACTCATGCTCA3', Ki autoantigen reverse: 5'TGTCCAAGCGTGCACACAT3'; TNF-α forward: 5'TGGAGAAGGGTGACCGACTC3', TNF-α probe: 5'CGCTGAGATCAATCGGCCCGACTAT3', and TNF-α reverse: 5'TCCTCACAGGGCAATGATCC3'. Primers and the probe for GAPDH were purchased from Applied Biosystems.

The Mann-Whitney U test was used to compare gene expressions in RA and OA samples. Spearman's rank coefficient was used to examine the relationship between expressions of 2 different genes, and the relationship between gene expressions and CRP, ESR, or RF. $P < 0.05$ was considered significant.

RESULTS

High expression of TTP gene in RA synovial tissue. Using DD-PCR, we selected and sequenced 105 genes from samples of one patient with RA. Identified genes were: complement C1r, ferritin L chain, collagen type 1, chitinase, TTP (G0S24), epididymal secretory protein, cytosolic selenium-dependent glutathione peroxidase, ubiquinol-cytochrome c reductase binding protein, NADH dehydrogenase subunit 2, 17-beta-hydroxysteroid dehydrogenase, IgG1 heavy chain, Ki autoantigen, CD63, sphingolipid activator, mortality factor 4, p47, cytochrome P450 IIIA4, and immunoglobulin-binding protein 1, others being either genes of unknown function or not in the databases. Among these genes, we focused on TTP, originally reported as a member of a set of genes (putative G0/G1 switch regulatory genes) that are expressed transiently on human PBMC, after addition of lectin or cycloheximide[10]. CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes were arbitrarily chosen for real-time PCR analyses, since we considered that the functions of these genes may also be related to the pathogenesis of RA. To determine whether TTP gene expression is indeed enhanced in RA synovial tissues, expression of TTP gene in synovial tissues of 22 RA and 22 OA patients was measured by TaqMan real-time semiquantification PCR. Expression of TTP gene was significantly higher in RA synovial tissues compared to OA synovial tissues ($p = 0.0128$, Mann-Whitney U test; Figure 1). Expression of CD63, immunoglobulin binding protein 1, mortality factor 4, and Ki nuclear autoantigen genes showed no significant differences between RA and OA synovium samples (data not shown).

TTP/TNF-α gene expression ratio correlates inversely with CRP. To determine whether the expression level of TTP

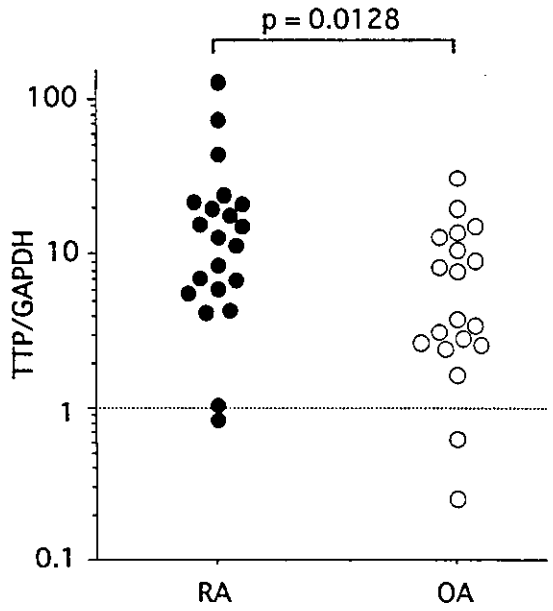

Figure 1. Expression of TTP mRNA in synovial tissues of patients with RA and OA. P value calculated by Mann-Whitney U test.

gene directly correlated with TNF-α gene, we analyzed expression of TNF-α gene using real-time semiquantification PCR, and the results were compared to the expression of TTP gene. Standard curves were generated that indicated the validity of the real-time semiquantification PCR used in this study (Figure 2). There was no significant correlation between the expression of TNF-α and TTP genes (Figure 3). To further examine the relevance of TTP gene expression in RA, we determined the relationships between TTP gene expression and CRP, ESR, or RF. CRP tended to be higher in patients with higher TNF-α gene expression, but without statistical significance (r = 0.306, p = 0.2071, Spearman rank correlation; Figure 4A). CRP also tended to be higher in patients with lower TTP gene expression, although this was statistically insignificant (r = –0.429, p = 0.0771, Spearman rank correlation; Figure 4B). However, when the ratio of TNF-α and TTP gene expression was calculated, and compared with CRP, a significant inverse relationship was observed (r = –0.653, p = 0.0071, Spearman rank correlation; Figure 5A). ESR showed a similar trend, although statistically insignificant (r = –0.441, p = 0.0692, Spearman

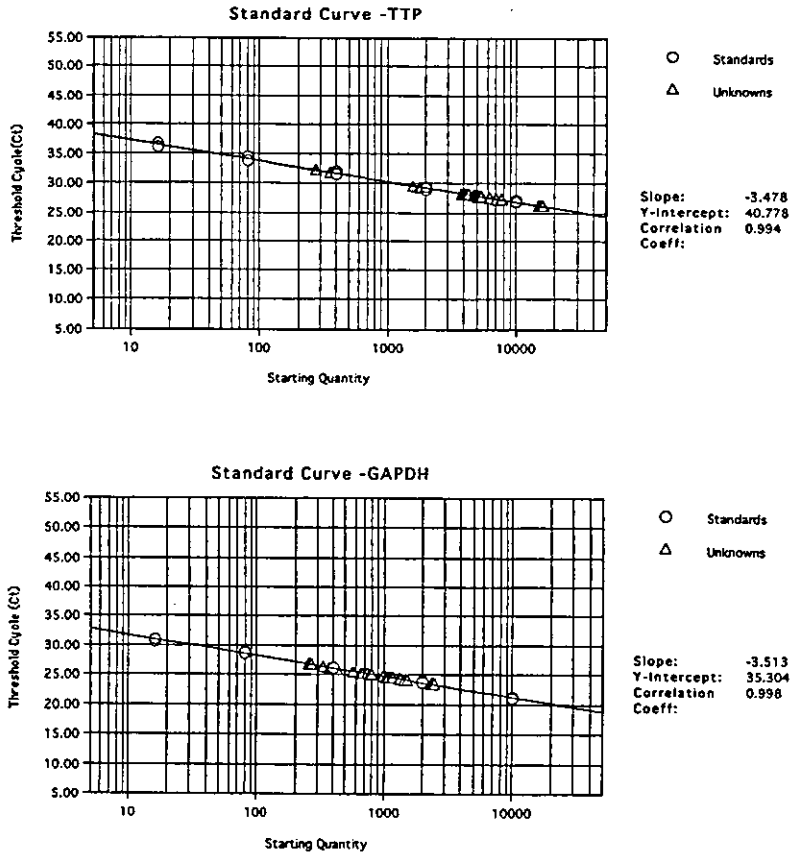

Figure 2. Standard curves generated for real-time semiquantification PCR. Upper panel: TTP; lower panel: GAPDH. All measurements were in triplicate, and sample values that were not within the standard curve were diluted and remeasured. Similar standard curves were obtained for all PCR analyses (data not shown).

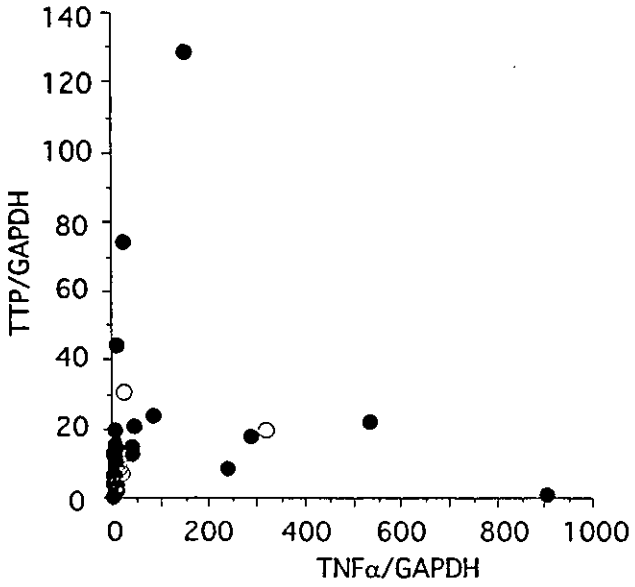

Figure 3. Relationship between expression of TTP mRNA and TNF-α mRNA. ●: RA, ○: OA.

rank correlation; Figure 5B). Steroid and/or DMARD usage and RF did not significantly correlate with TTP gene expression (data not shown).

DISCUSSION

Our results suggest that TTP gene expression may play an important role in RA disease activity. Elucidation of the role of TTP in the pathogenesis of RA may be helpful in the search for new therapies for RA.

DD-PCR is a powerful tool for identifying genes that are highly expressed in one of the 2 samples compared. Using this technique, we identified TTP as a possible candidate gene whose expression may have a role in the pathogenesis of RA. Our strategy was to compare samples from the same patient, to avoid detecting differences between individuals, then use real-time PCR to determine whether the identified genes are highly expressed in synovial tissues from RA patients compared to those from OA patients. Applying DD-PCR to synovial tissues from RA and OA samples is an another possible approach, which may give us a completely different result.

We found that the expression of TTP gene was significantly higher in synovial tissues from RA patients than those from OA patients. In addition, in RA patients the magnitude of TTP gene expression was lower in patients with higher serum CRP, an inflammation marker commonly used to monitor RA activity. Although this relationship was not quite statistically significant ($p = 0.0771$), a significant relationship was observed when TNF-α gene expression was taken into account ($p = 0.0071$). A similar relationship for ESR was also observed ($p = 0.0692$), although it was not

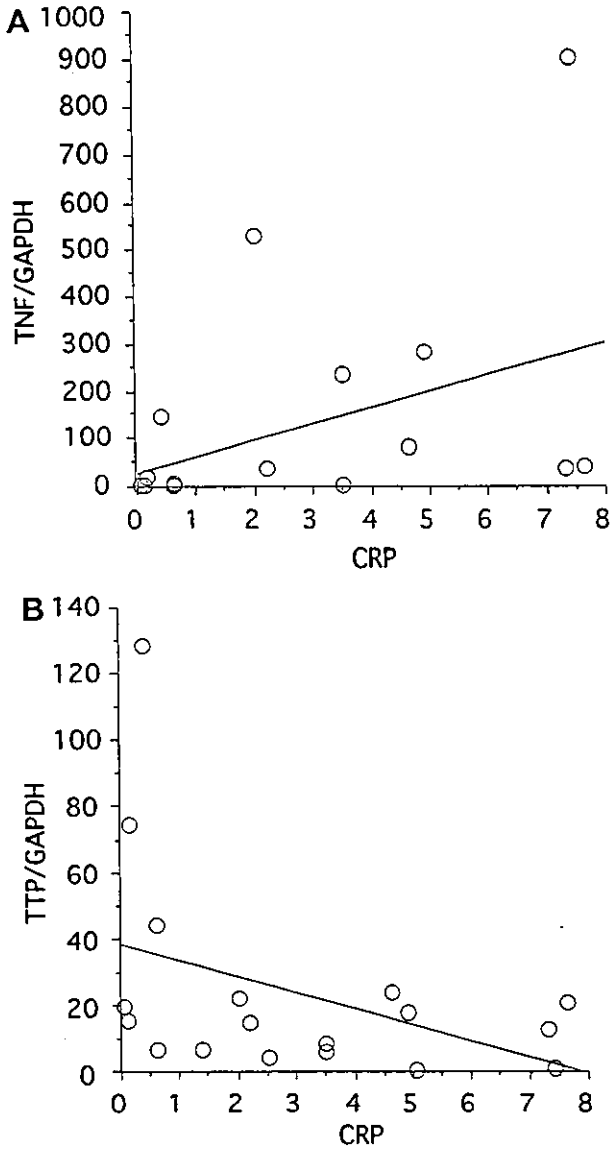

Figure 4. Relationship between serum CRP and expressions of TNF-α mRNA and TTP mRNA. A. CRP and TNF-α mRNA ($p = 0.2071$, Spearman rank correlation). B. CRP and TTP mRNA ($p = 0.0771$, Spearman rank correlation).

statistically significant. The finding that TTP gene expression is higher in RA samples is not merely due to more inflammatory cells in RA samples than OA samples, although this may partly account for the finding. If enhanced TTP gene expression reflects only the increment of inflammatory cells in the synovium, one would expect that, in RA synovium, TTP gene expression would correlate with TNF-α gene expression and inflammatory markers. Such a relationship was not observed. Instead, TTP gene expression seemed to be lower in synovium from patients with more active inflammation.

TTP protein binds directly to the AU-rich element in the

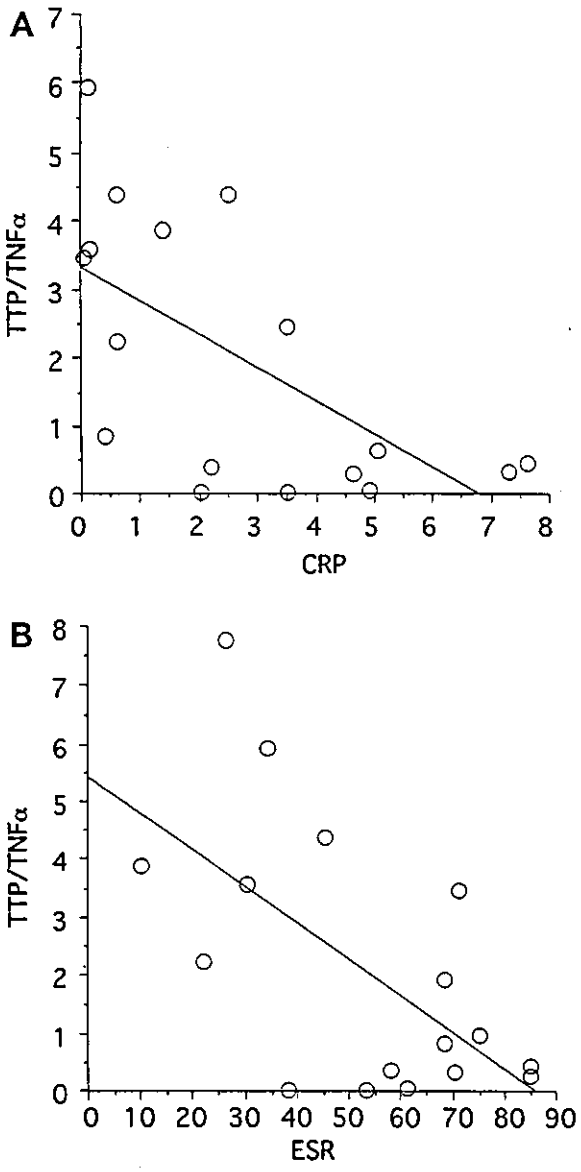

Figure 5. Relationship between expression of TTP/TNF-α gene expression ratio and CRP or ESR. A. TTP/TNF-α gene expression ratio and CRP (r = –0.653, p = 0.0071, Spearman rank correlation). B. TTP/TNF-α gene expression ratio and ESR (r = –0.441, p = 0.0692, Spearman rank correlation).

3'-UTR of TNF-α mRNA. Since binding of TTP leads to instability of TNF-α mRNA[8,11], it is conceivable that TTP is a physiological regulator of TNF-α production. It has been reported that TTP knockout mice develop erosive arthritis, dermatitis, conjunctivitis, glomerular mesangial thickening, and high titers of anti-DNA and antinuclear antibodies[12]. The pathological findings of erosive arthritis were similar to those observed in human RA. The phenotype seen in TTP knockout mice was reversed by administration of anti-TNF-α antibody[12], suggesting that TNF-α overproduction plays a major role in the pathogenesis of arthritis and other symptoms in these mice. In addition, TTP binds to AU-rich elements on mRNA of other genes such as interleukin 2 (IL-2), IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF), and c-fos genes[13]. In T cells, TTP protein is increased after cell stimulation, and replaces HuA, an AU-rich element-binding protein, which stabilizes mRNA[13]. Thus, TTP is a natural suppressor of excessive cytokine production. We speculate that in patients with RA, the presence of TNF-α in synovial tissues could lead to enhancement of TTP production, and that patients who lack the potential to produce adequate amounts of TTP may develop more severe disease, leading to more intense inflammation and joint destruction. The level of TTP gene expression in the synovial tissue may be important in determining the disease activity of RA, making it a possible candidate for future therapeutic targets.

The lack of correlation between TTP mRNA and the amount of TNF-α mRNA does not necessarily indicate that the expression of TTP mRNA is not an important factor in TNF-α production in the synovial tissue of patients with RA. Physiologically, overproduction of TNF-α would lead to enhanced production of TTP, which in turn would suppress TNF-α production. On the other hand, inadequate production of TTP in RA patients would lead to overproduction of TNF-α, affecting the course of RA. Thus, the relationship between TTP mRNA production and TNF-α production may differ among individual patients. Indeed, the significant inverse relationship between TTP/TNF-α gene expression ratio and CRP suggests that an adequate TTP response could help in the control of inflammation that occurs in RA synovial tissues. Brooks, *et al*[14] reported the presence of TTP protein in RA synovial tissue. They also reported that human TTP binds to the 3'-UTR of TNF-α mRNA and reduces reporter gene expression. Their study emphasized the potential importance of posttranscriptional regulation of TNF-α production in the pathogenesis of RA. Our findings add support to their conclusions. Our study was done using synovial samples; it may be of interest to investigate whether a similar relationship can be observed in peripheral blood samples from RA patients. Studies at the protein level also should be done.

Our study does not indicate that elevated TTP gene expression is an RA-specific phenomenon. TNF-α is known to play important roles in various inflammatory diseases such as psoriatic arthritis, ankylosing spondylitis, Behçet's disease, and Crohn's disease. It is possible that interindividual differences in TTP production affect the arthritic condition in these diseases. TNF-α expression in cartilages from patients with OA has also been reported[15]. Thus, TNF-α and hence TTP may have a role in the progression of OA as well as RA. Whether TTP gene expression is elevated in OA synovium compared to healthy synovium, and whether this has implications for the severity of OA, are challenging

questions because of the difficulties of obtaining healthy synovial samples and assessing the severity of OA.

We speculate that TTP production is induced in patients with RA as a negative feedback of TNF-α overproduction, and that TTP may affect the course of RA by reducing the production of TNF-α in the synovium. Our current hypothesis is that compounds that mimic the properties of TTP or that enhance TTP gene expression, or even TTP gene therapies, may serve as a tool for controlling joint inflammation and destruction in severe RA.

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Polymorphisms of IL-1ß Gene in Japanese Patients with Sjögren's Syndrome and Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Interleukin (IL)-1ß is a proinflammatory cytokine involved in various immune responses. Five polymorphisms in the IL-1ß gene have been described, and relationships between these polymorphisms and some autoimmune diseases have been reported. Evidence suggests that IL-1ß may be involved in the destruction of salivary and lacrimal glands in Sjögren's syndrome (SS). We evaluated the significance of IL-1ß gene polymorphisms in SS.

Methods. Blood samples were taken from 101 patients with SS, 103 patients with systemic lupus erythematosus (SLE, excluding those with secondary SS), and 106 healthy volunteers. Each polymorphism of the IL-1ß gene was analyzed by polymerase chain reaction (PCR) amplification of the polymorphic site, followed by site-specific restriction digestion. Genotype frequencies of each polymorphism in SS patients were compared with those of the controls and SLE patients, and differences between primary and secondary SS patients were also compared.

Results. Genotypes CC, TT, and AA in positions –511, –31, and 3877, respectively, were significantly less frequent in SS patients than controls or patients with SLE. No significant differences were found in genotype frequencies of any of the polymorphisms between patients with primary SS and secondary SS.

Conclusion. IL-1ß gene polymorphisms may affect susceptibility to SS, but not SLE. (J Rheumatol 2004;31:720–5)

Key Indexing Terms:
INTERLEUKIN 1ß
GENE POLYMORPHISM
SJÖGREN'S SYNDROME
SYSTEMIC LUPUS ERYTHEMATOSUS

Interleukin (IL)-1ß is a proinflammatory cytokine involved in various immune responses, including autoimmune diseases[1]. The gene is located on chromosome 2q 13[2], and 5 polymorphisms have been described in the promoter regions (at –511: C/T)[3,4], TATA Box (at –31: T/C)[3,5], exon 4 (at +3263: C/T)[3,6], intron 4 (at +3877: A/G)[3,5], and exon 5 (at +3953: C/T)[3,7]. At least 3 of these, –511[8], –31[9], and +3953[7], are possibly related to IL-1ß production. It has been shown that the presence of the T allele at +3953 has a stimulatory effect on lipopolysaccharide (LPS)-induced IL-1ß protein production *in vitro*[7]. Santtila, *et al*[8] found that mononuclear cells derived from individuals with the T allele in the polymorphism at –511 and those without T allele in the polymorphism at 3953 showed slightly higher production of IL-1ß than other carriers, when stimulated by phorbol dibutyrate. The T allele at –31 of the IL-1ß gene comprises a TATA box-related gene structure, suggesting a role in gene expression[9].

Recent studies have described the relationships between these polymorphisms and various diseases such as rheumatoid arthritis (RA)[10-13], osteoporosis[6], osteoarthritis[14], inflammatory bowel diseases[15], gastric cancer[9], gastritis caused by *Helicobacter pylori*[16], multiple sclerosis (MS)[17], and alcoholic liver disease[18]. In autoimmune diseases, Buchs, *et al*[11] showed that patients with the T allele at +3953 show aggressive destructive arthritis compared to those without the T allele. On the other hand, Niino, *et al*[17] reported the lack of significant differences in the distribution of polymorphisms between Japanese patients with MS

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and controls. In addition, in their study, no association was observed between IL-1ß or IL-1ra gene polymorphisms and clinical characteristics. Huang, *et al*[19] also reported that the polymorphisms on promoter and exon 5 regions of the IL-1ß gene are not related to SLE in Taiwanese patients.

Sjögren's syndrome (SS) is an autoimmune disease characterized by multisystem inflammation mainly affecting the exocrine glands. There are common features between SS and systemic lupus erythematosus (SLE), but compared to SLE, primary SS (pSS) is a more organ-specific disease. Infiltrating mononuclear inflammatory cells inhibit the function of glandular cells in the salivary and/or lacrimal gland tissues. The typical symptoms of SS, dry eye (keratoconjunctivitis sicca) and dry mouth occur following destruction of these glands[20]. IL-1ß and tumor necrosis factor (TNF)-α are proinflammatory cytokines and seem to be involved in the destruction of acinar structure in human salivary glands[21,22]. In addition, IL-1ß may play a key role in the pathogenesis of keratoconjunctivitis sicca[23]. Although several genetic studies of autoimmune diseases including studies on cytokine polymorphisms, have already been reported[24], the relationships between IL-1ß gene polymorphisms and occurrence of SS have not been examined, and only one study is reported for SLE[19]. To investigate the effect of IL-1 gene polymorphisms on the susceptibility to SS, we analyzed the gene polymorphisms of IL-1ß by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method, and compared the genotype frequencies of each polymorphism between SS and controls, SLE and controls, or SS and SLE. In addition, detailed analysis evaluated whether these polymorphisms affect the phenotypes of SS. Finally, we studied the relationships between gene polymorphisms and characteristics of these diseases.

MATERIALS AND METHODS

SS patients, SLE patients and healthy controls. Approval for this study was obtained from the local ethics committee and written informed consent was obtained from all patients and volunteers who participated in this study. Blood samples were collected from 101 Japanese patients with SS and 103 Japanese patients with SLE followed at the Department of Internal Medicine, University of Tsukuba Hospital. All SS patients [43 with primary SS, 58 with secondary SS including 21 with rheumatoid arthritis, 20 with SLE, 11 with SSC, 4 with mixed connective tissue disease (MCTD), and 2 with Hashimoto's disease] satisfied the Japanese Ministry of Health criteria for the classification of SS[25], and all SLE patients (no patients with secondary SS, 10 men and 93 women) satisfied the 1997 revised American College of Rheumatology criteria for SLE[26]. As healthy controls, 106 Japanese volunteers (41 men, 65 women) were recruited from our institute.

IL-1ß polymorphism typing. Genomic DNA was isolated from 0.5 ml of anti-coagulated peripheral blood by using DNA QuickII DNA purification kit (Dainippon Pharmaceuticals, Osaka, Japan) and the instructions supplied with the kit. Five polymorphisms of the IL-1ß gene were analyzed by PCR-RFLP[4-6,13]. Briefly, 4 sets of PCR reactions were performed, and the amplified products were digested with appropriate restriction enzymes that cut one of the alleles in each polymorphism. The primers and restriction enzymes for each polymorphism were as follows. For -511: 5'-TGGCATTGATCTGGTTCATC-3', 5'-GTTTAGGAATCTTCCCACTT-3', and *Ava* I. For -31: 5'-TCATAGTTTGCTACTCCTTGC-3', 5'-CAAAAAGCTGAGAGAGGAGG-3', and *Alu* I. For +3263: 5'-TTTGAGAGGCAGGCTGTTTG -3', 5'-CTTGGTTGTCTTCCTACTATG-3' and *Mbo* II. For +3877 and +3953: 5'-GTTGTCATCAGACTTTGACC-3', 5'-TTCAGTTCATATGGACCAG-3', *BsoF* I for +3877 and *Taq* I for +3953. Digested fragments were run on 2.5% agarose gels and visualized with ethidium bromide (Figure 1).

Serum levels of IL-1ß. IL-1ß levels were measured with human IL-1ß US ELISA kit (BioSource International, Inc. Camarillo, CA, USA), using the instructions provided by the manufacturer.

Statistical analyses. Linkage disequilibrium analysis of these polymorphic sites in the IL-1ß gene was performed using Graphical Overview of Linkage Disequilibrium (GOLD) software package[27]. Chi-square analysis was used to compare genotypes or frequencies of alleles and phenotypes between patients and control groups. Fisher's exact test was used where appropriate. A p value of 0.05 was considered significant. For multiple comparisons (SS or pSS, healthy controls. SLE), a p value of 0.016 was considered as significant. Odds ratios were also calculated for disease susceptibility in carriers of a specific genotype. Correlations between each genotype in each polymorphism and clinical and biological variables were evaluated by analysis of variance or chi-square analysis.

RESULTS

Validation of the RFLP analyses. Fragments of predicted sizes were obtained for all polymorphisms (Figure 1), except for position +3263, where all samples were digested by *Mbo* II, indicating the absence or extreme rarity of the T allele in our Japanese population. Several randomly selected samples were sequenced to further confirm the accuracy of the RFLP analysis (data not shown).

Relationship between IL-1ß genotypes and SS and SLE. Comparison of SS group with other groups showed no evident differences in allele frequencies at any of polymorphisms (data not shown; can be calculated from Table 1A). In contrast, comparison of SS patients with other groups showed significant differences in genotype distribution at positions -511, -31, and 3877 (-511CC/CT/TT: SS vs controls and SLE: p = 0.053 and p = 0.028; -31 TT/TC/CC: SS vs SLE: p = 0.027; 3877 AA/AG/GG: SS vs controls and SLE: p = 0.039 and p = 0.054) (Table 1A). Specifically, the frequencies of genotypes CC, TT, and AA at positions -511, -31, and 3877, respectively, were lower in SS patients compared with the controls and the SLE group (-511, -31, 3877: SS/HC/SLE:18.8, 19.6, 22.8%; 32.1, 29.2, 36.8%; 34.0, 32.4, 35.9%). These differences were also evident when patients were categorized according to the presence or absence of these genotypes (-511CC/CT+TT: SS vs control and SLE: p = 0.029 and p = 0.014; -31 TT/TC+CC: SS vs control and SLE: p = 0.111 and p = 0.041: 3877 AA/AG+GG: SS vs control and SLE: p = 0.028 and p = 0.039, respectively, Table 1B). After adjustment for multiple comparisons according to Bonferroni, only the difference at 511 between SS and SLE remained significant. No significant differences were present between the SLE patients and controls (Table 1A-B). No significant differences in genotype frequencies were observed between primary and secondary SS (Table 1A). In agreement with previous

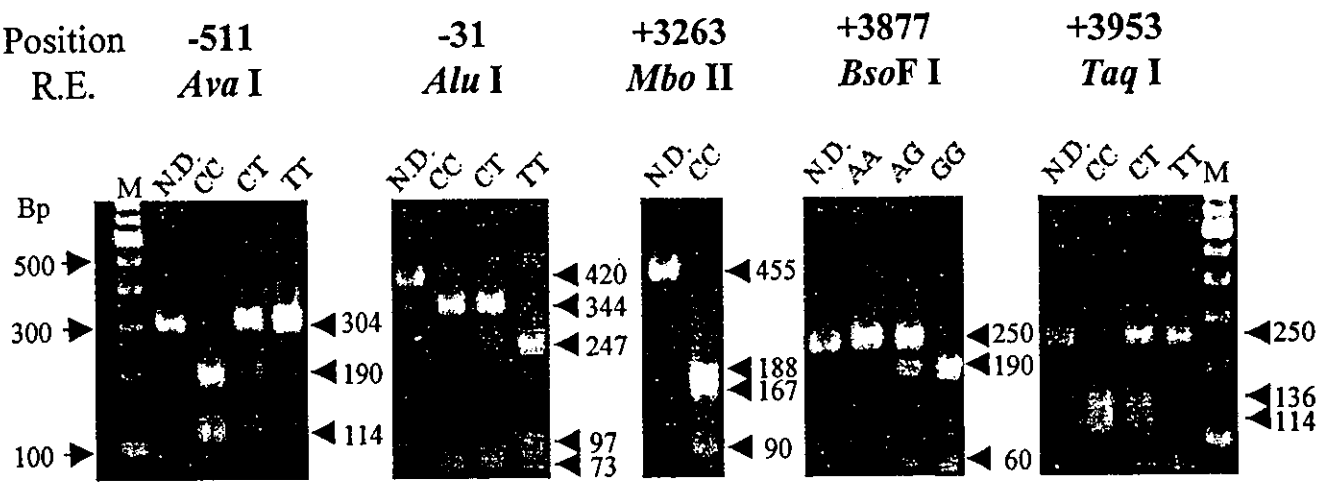

Figure 1. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the 5 polymorphisms of the IL-1ß gene. cDNA was amplified with primers indicated in the text, and restriction digested by appropriate enzymes. Digested PCR products were run on agarose gels and visualized with ethidium bromide. RE: restriction enzyme. M: 100 bp marker. ND: non-digested PCR product.

Table 1A. Genotype distribution of IL-1ß gene polymorphisms in patients with SS and SLE and healthy controls.

Polymorphism	Genotype	HC n = 106 (%)	SS n = 101 (%)	pSS n = 43 (%)	sSS n = 58 (%)	SLE n = 103 (%)	p (chi-square)
	CC	34 (32.1)	19 (18.8)	7 (16.3)	12 (20.7)	35 (34.0)	
-511	CT	49 (46.2)	62 (61.4)	26 (60.5)	36 (62.1)	46 (44.7)	0.053
	TT	23 (21.7)	20 (19.8)	10 (23.3)	10 (17.2)	22 (21.4)	0.028*
	TT	31 (29.2)	19 (19.6)	7 (17.5)	12 (21.1)	33 (32.4)	
-31+	TC	52 (49.1)	57 (58.8)	23 (57.5)	34 (59.6)	41 (40.2)	0.246
	CC	23 (21.7)	21 (21.6)	10 (25.0)	11 (19.3)	28 (27.5)	0.027*
	AA	39 (36.8)	23 (22.8)	10 (23.3)	13 (22.4)	37 (35.9)	
3877	AG	48 (45.3)	63 (62.4)	24 (55.8)	39 (67.2)	50 (48.5)	0.039
	GG	19 (17.9)	15 (14.9)	9 (20.9)	6 (10.3)	16 (15.5)	0.054*
	CC	98 (92.5)	92 (91.1)	40 (93.0)	52 (89.7)	93 (90.3)	
3953	CT	8 (7.5)	9 (8.9)	3 (7.0)	6 (10.3)	9 (8.7)	
	TT	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.0)	

SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; HC: healthy control; pSS: primary SS; sSS: secondary SS; +: 4 SS patients and 1 SLE patient were not genotyped. p: SS vs HC; *: SS vs SLE. p values of ≤ 0.016 were considered significant after Bonferroni corrections.

Table 1B. Genotype skewing of IL-1ß gene polymorphisms in patients with SS and SLE and healthy controls.

Polymorphism	Phenotype	HC n = 106 (%)	SS n = 101 (%)	pSS n = 43 (%)	SLE n = 103 (%)	p (chi-square)	p (Fisher's probability test)	OR (95% CI)
						0.029‡	0.038	0.49 (0.26–0.93)
-511	CC	34 (32.1)	19 (18.8)	7 (16.3)	35 (34.0)	0.053*	0.068*	0.41 (0.17–1.02)
	CT/TT	72 (67.9)	82 (81.2)	36 (83.7)	68 (66.0)	0.014**	0.017**	0.45 (0.24–0.86)
						0.031***	0.044***	0.38 (0.15–0.94)
	TT	31 (29.2)	19 (19.6)	7 (17.5)	33 (32.4)	0.111	0.142	0.59 (0.31–1.13)
-31+	TC/CC	75 (70.8)	78 (80.4)	33 (82.5)	69 (67.6)	0.041**	0.053**	0.51 (0.27–0.98)
	AA	39 (36.8)	23 (22.8)	10 (23.3)	37 (35.9)	0.028	0.034	0.51 (0.28–0.93)
3877	AG/GG	67 (63.2)	78 (77.2)	33 (76.7)	66 (64.1)	0.039**	0.046**	0.53 (0.28–0.97)
	CC	98 (92.5)	92 (91.1)	40 (93.0)	93 (90.3)			
3953	CT/TT	8 (7.5)	9 (8.9)	3 (7.0)	10 (9.7)			

SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; HC: healthy control; pSS: primary SS; OR: odds ratio; 95% CI: 95% confidence interval; +: 4 SS patients and 1 SLE patient were not genotyped; ‡ SS vs HC; * pSS vs HC; ** SS vs SLE; *** pSS vs SLE. p values of ≤ 0.016 were considered significant after Bonferroni corrections.

reports[9,16], –511 and –31 polymorphisms were in almost complete linkage disequilibrium (Table 1A-B). Detailed analysis of linkage disequilibrium among the 4 polymorphisms examined in this study showed that these polymorphisms are indeed in linkage disequilibrium (D'= 0.622~1.000), and are not independent of each other.

Relationships between IL-1ß genotypes and serum IL-1ß. Serum IL-1ß was measured in 84 healthy controls, 43 patients with SS, and 101 patients with SLE. In these groups, measurable amounts of serum IL-1ß were detected in 11 controls, 4 patients with SS, and 5 patients with SLE. IL-1ß concentration in other samples did not exceed the detection limit (0.3 pg/ml). IL-1ß genotypes in individuals with measurable serum IL-1ß did not show any skewing toward a particular genotype (data not shown).

Relationships between IL-1ß genotypes and disease characteristics of SS and SLE. Disease characteristics and variables were examined in the context of IL-1ß genotypes in SS and SLE patients. Presence of anti-SSA antibody was slightly increased in patients with genotype CC and genotype TT at positions –511 and –31, respectively, ($p = 0.040$ and $p = 0.025$, Table 2). The presence of antinuclear antibody or anti-SSB antibody was not related to any of the genotypes examined (Table 2). Age at onset and presence of arthritis were also not associated with any of the genotypes examined (data not shown). In SS patients, serum IgG or presence of sicca syndrome was not associated with any of the IL-1ß genotypes studied (data not shown). In SLE patients, no significant relationship was observed between IL-1ß genotypes and disease variables or presence of symptoms including SLE Disease Activity Index[28], anti-DNA antibody, C_3, C_4, CH50, central nervous system lupus, serositis, and glomerulonephritis (data not shown).

DISCUSSION

Our major finding was that the genotype frequencies and/or the genotype skewings of 3 (–511, –31, +3877) of the 5 known IL-1ß gene polymorphisms were significantly different between SS patients and controls or SLE subjects. No such difference was observed between SLE patients and controls, which is in accord with a report on Taiwanese SLE patients [19]. In fact, in our study, significant differences were observed between patients with SS and SLE. Two (–511, –31) of these 3 polymorphisms were on the promoter region of the IL-1ß gene, and thus may affect IL-1ß gene expression and subsequent cytokine production. The third (+3877) was on intron 4, and we cannot speculate whether this polymorphism influences IL-1ß production or some other immunological or inflammatory functions, although this polymorphism is suggested to be an informative marker for disease association studies[5]. Although many of the significant differences were lost after adjustment of the p value for multiple comparisons, our results may provide a rationale for further studies on the role of the IL-1ß and the pathogenesis of SS. Analysis of linkage disequilibrium among the 4 polymorphisms observed in this study showed that these polymorphisms are in linkage disequilibrium and are not independent, and therefore, adjustment for multiple comparison was performed for comparisons between patients and healthy controls.

In SS, salivary and lacrimal glands are the main target organs for autoimmune tissue damage. IL-1ß and TNF-α are proinflammatory cytokines, and affect the destruction of acinar structure in salivary glands[21]. Messenger RNA of both Th1 and Th2 cytokines are detected in salivary glands of patients with SS, and may have important roles in the onset and progression of SS[22,29,30]. Interferon-γ, IL-2, IL-6, IL-10, and transforming growth factor-ß play essential roles in the induction of SS, while IL-4 and IL-5 may be involved in the progression of disease process[29]. IL-1ß has multiple biologic effects, which include induction of lymphokine synthesis (IL-2, -3, -4, -5, -6, -7, -10, and -12), and devel-

Table 2. Comparison of genotype distribution between patients positive for autoantibodies (ANA, SSA, SSB) and negative in SS and SLE.

Polymorphism	Genotype	ANA				Anti-SSA				Anti-SSB			
		SS (n = 97)		SLE (n = 89)		SS (n = 81)		SLE (n = 86)		SS (n = 65)		SLE (n = 85)	
		+	–	+	–	+	–	+	–	+	–	+	–
	CC	16	3	26	5	15	1	6	23	0	15	0	28
–511	CT	43	16	33	6	29	18*	10	31	7	29	0	41
	TT	15	4	16	3	14	4	6	10	2	12	1	15
	TT	16	3	26	4	15	1	6	22	0	15	0	27
–31	TC	41	14	27	6	26	18**	9	26	6	28	0	35
	CC	15	5	21	4	14	4	7	15	2	12	1	21
	AA	16	7	28	2	14	4	5	23	0	14	0	27
3877	AG	47	13	35	9	33	17	11	32	6	33	1	42
	GG	11	3	12	3	11	2	6	9	3	9	0	15
	CC	67	21	69	12	52	22	19	59	8	50	1	76
3953	CT	7	2	6	1	6	1	2	5	1	6	0	7
	TT	0	0	0	1	0	0	1	0	0	0	0	1

ANA: antinuclear antibody; SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; * p = 0.040; ** p = 0.025 (n = 78), by chi-square test.

opment of Th2 type T cells[1]. IL-1 is known to initiate transcription and stabilize the mRNA of a variety of genes. Messenger RNA expression of inflammatory cytokines, such as IL-6 and 8, TNF-α, and granulocyte-macrophage colony-stimulating factor genes are upregulated by IL-1[1,31,32]. In this study, we found that the genotype skewings of 3 polymorphisms of the IL-1ß gene were significantly different in SS patients compared to those with SLE or controls. While adjustment for multiple comparison leads to the loss of statistical significance in many of the differences observed, we still believe that our data are of interest, since our primary focus was on SS, which has evidence suggesting the importance of IL-1ß in its pathogenesis, and not on SLE, which has no such evidence and which was included in this study rather as a comparator disease. We speculate that these polymorphisms may have direct or indirect impact on the onset or progression of SS.

In previous reports on IL-1ß polymorphisms and IL-1ß production, the relationship between the polymorphism and IL-1ß gene expression or IL-1ß production is not fully determined[8,33]. In our study, although we tried to evaluate the relationship between these polymorphisms and serum IL-1ß we could not find any relationship between the 4 polymorphisms of IL-1ß gene and concentration of serum IL-1ß, since only 11 individuals in the control group, 4 in the SS group, and 5 in the SLE group had measurable amounts of serum IL-1ß. These results were not unexpected, since it is difficult to detect IL-1ß in serum or plasma without *in vitro* stimulation by mitogens such as LPS because circulating levels of IL-1ß are very low and plasma normally contains IL-1sRII (IL-1 soluble receptor II), α-2-macroglobulin and complement, which bind to IL-1ß[1]. In addition, in most of our patients, disease activity was adequately controlled at the time of sampling. Therefore, to assess whether these polymorphisms are indeed associated with IL-1ß production, either a study with a large number of untreated patients or an *in vitro* study using a promoter assay would be necessary.

Genotypes at –511 and –31 of the IL-1ß gene were in almost complete linkage disequilibrium as reported by others[9,16]. Therefore, SS patients showed similar significant decreases of CC homozygous genotype at –511 and of TT homozygous genotype at –31. Presence of the T allele at –31 would make a TATA box-related sequence, which speculatively may lead to increased IL-1ß production. However, the number of individuals who are TT homozygous at –31 was significantly decreased in the SS patient group. Since the presence of this genotype was tightly linked with the probable high IL-1ß producing allele at –511[9,16], the significance of TATA box formation at –31 cannot be determined at this stage.

Polymorphism at +3877 in intron 4 of the IL-1ß gene, originally reported by Guasch, *et al*[5] was proposed as an adequate marker for genotype-disease association studies, because of its heterogeneity and low linkage to other polymorphisms. However, Langdahl, *et al*[6] reported the lack of any difference in genotype distributions at +3877 of the IL-1ß gene, or at +11100 of the IL-1ra gene between healthy controls and patients with osteoporosis. Furthermore, they found no relationships between these polymorphisms and disease variables of osteoporosis such as bone mass and bone turnover. In our study, we found that the G allele carriers at +3877 of the IL-1ß gene are significantly more frequent among SS patients compared to healthy controls or SLE patients. Since there is no information on the relationship between this polymorphism and gene expression, the significance of this finding remains unclear at present.

We found no significant skewing of genotype distribution in any of the polymorphisms studied with any of the disease characteristics including age at onset, severity of sicca syndrome, presence or absence of arthritis, anti-SSB antibody, and antinuclear antibody in both SS and SLE patients. The CC homozygous genotype at –511 and TT homozygous genotype at –31 were slightly increased in anti-SSA antibody-positive patients, compared to negative patients. Although IL-1ß and anti-SSA antibody production may be indirectly associated in some way in the complex steps of autoantibody production, considering the number of comparisons made in this study, this weak association should be retested in future studies.

Finally, the IL-1ß gene allele frequencies at positions –511 and +3953 observed in our study controls were similar to those obtained in healthy Japanese subjects studied by Takamatsu, *et al*[18] and in healthy Taiwanese subjects studied by Huang, *et al*[19], but were different from those obtained in healthy Caucasian subjects studied by Cantagrel, *et al*[13] because the allele T on +3953 is much more common in Caucasians than in the Japanese (frequency of TT homozygous healthy individuals was 0% in our study and in Takamatsu's[18], but was 4.7% in Cantagrel's[13]).

There are important steps, e.g., IL-1ß converting enzyme (ICE) digestion, competition with IL-1ra and IL-1a, and binding with the IL-1 receptor, before IL-1ß gene products exert their functions. Other cytokines and mediator molecules also affect this cascade. Therefore, the extent of IL-1ß function is dependent not only on IL-1ß gene expression. Nevertheless, our results showed significant differences in genotype distributions in 3 polymorphisms of the IL-1ß gene in SS patients compared with controls or patients with SLE. These findings imply that IL-1ß gene polymorphisms, and hence, IL-1ß function, may influence the onset and progression of SS. On the other hand, no such genotype skewing was observed in SLE patients, suggesting that this cytokine might not be an important factor in the onset of SLE.

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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).

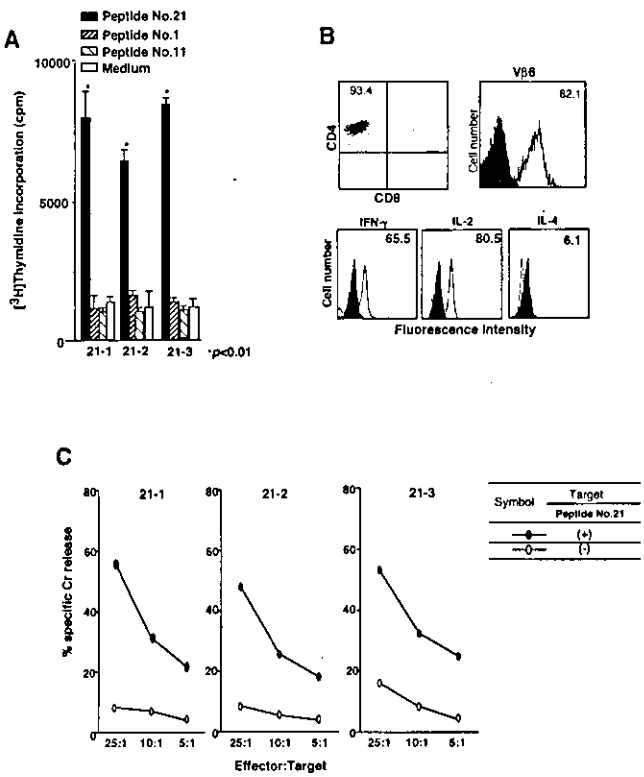

Figure 1. Establishment of autoreactive T cell lines. **A,** Three strongly proliferative T cell lines (21–1, 21–2, 21–3) from JS-1 peptide p21–stimulated lymph node cells were isolated and ^3H-thymidine incorporated measured. Values are the mean and SEM. **B,** Flow cytometric analysis, showing that 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-

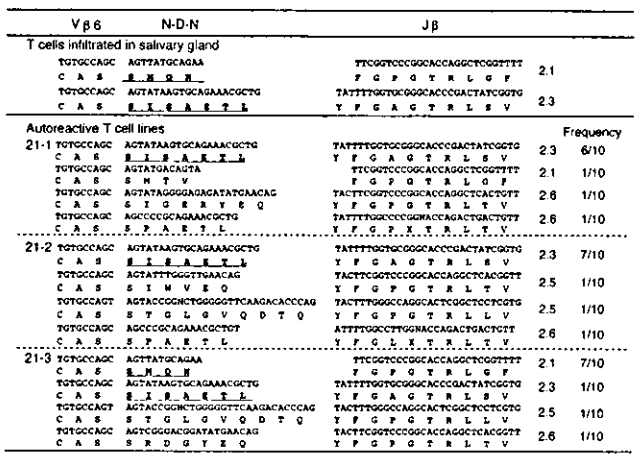

Figure 2. T cell receptor V_β gene usage and third complementarity-determining region (CDR3) sequences of infiltrating T cells in NFS/*sld* mice that had undergone thymectomy on day 3 after birth and of α-fodrin p21–specific lines (21–2, 21–2, 21–3), determined by reverse transcriptase–polymerase chain 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

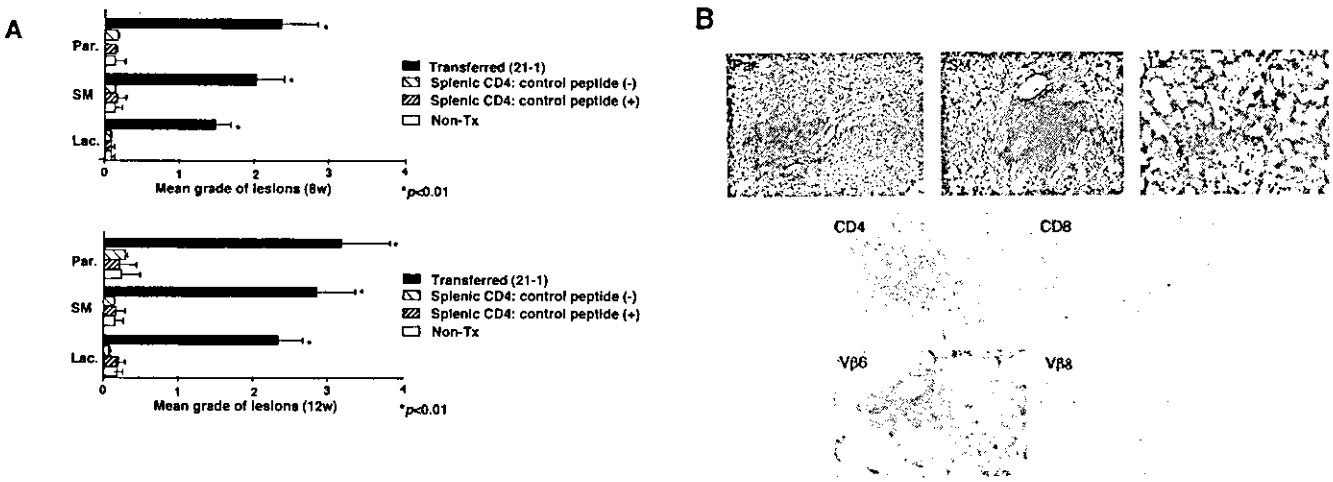

Figure 3. Adoptive transfer of autoreactive T cell line 21–1 into normal syngeneic recipients. **A,** Autoimmune lesions in the salivary and lacrimal (Lac.) glands developed at 8 weeks (n = 7) and 12 weeks (n = 8) after intraperitoneal injection with cells from T cell line 21–1 (1×10^6) into irradiated (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.

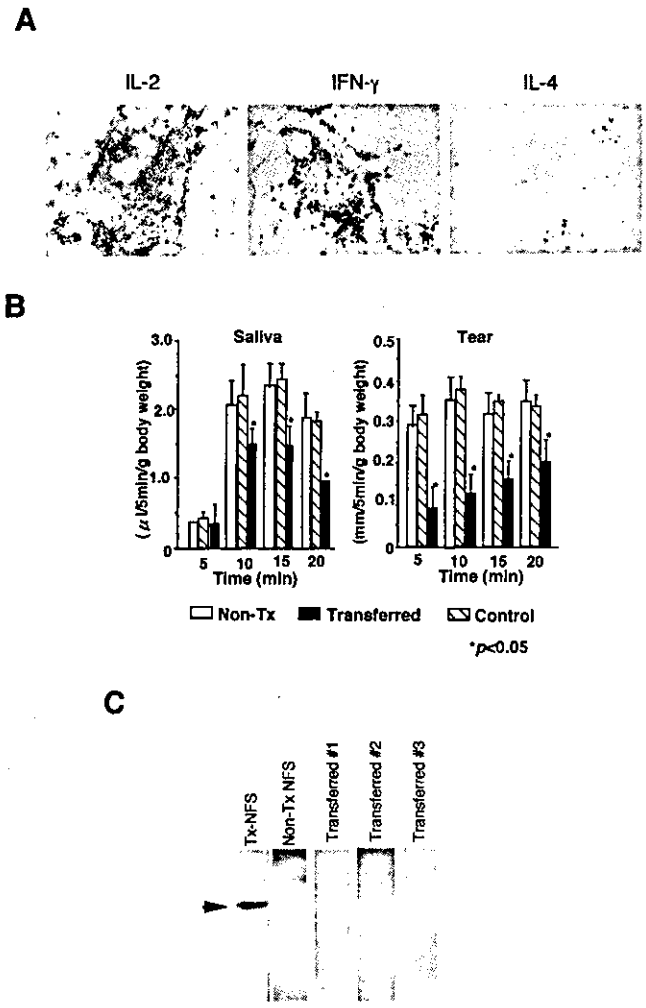

Figure 4. A, Immunohistologic features in adoptively transferred mice. A large proportion of infiltrating cells in the salivary glands were positive for Th1 cytokines such as interleukin-2 (IL-2) and interferon-γ (IFNγ), but not IL-4. **B,** Mean and SEM saliva and tear volume (n = 5 per group) in adoptively 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-

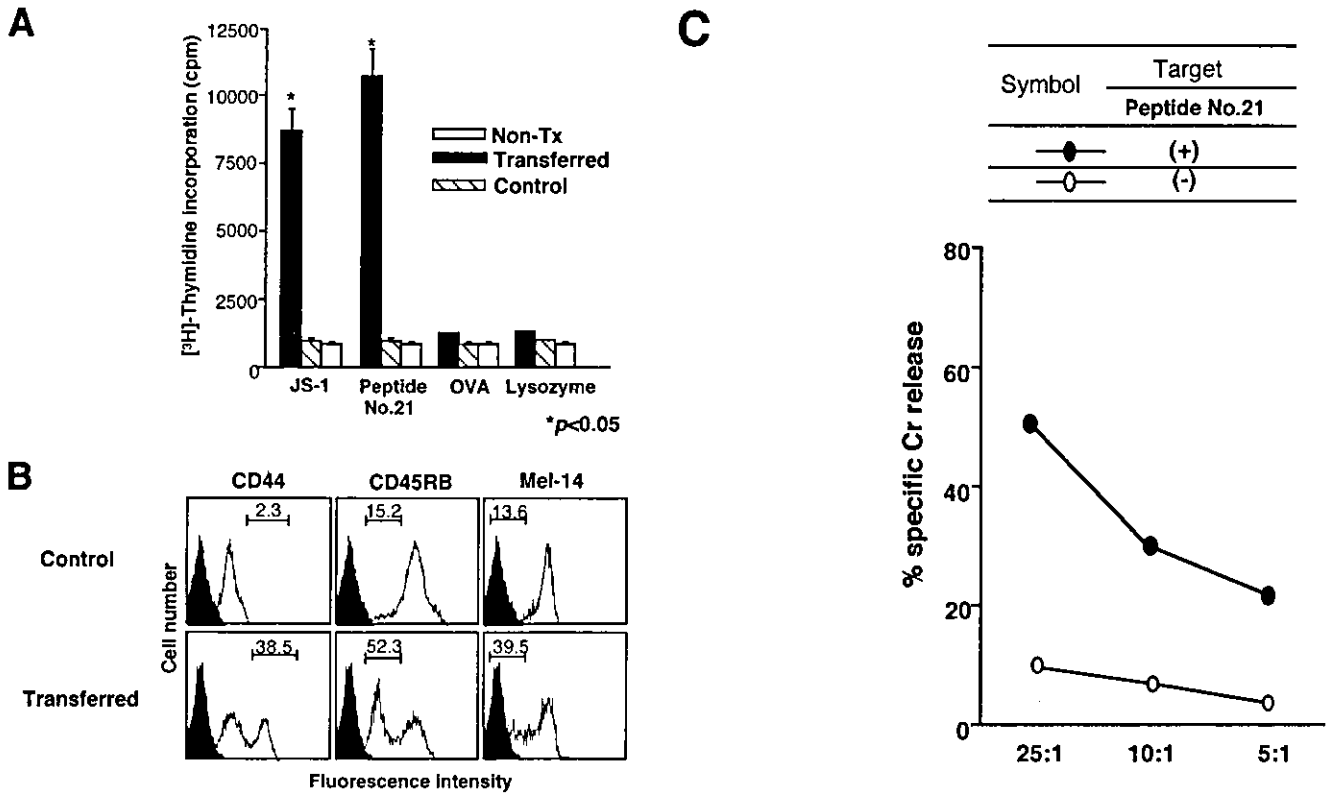

Figure 5. A, T cell proliferation in spleen cells. Adoptively transferred mice showed a significant increase of autoantigen (JS-1 and peptide 21)–specific T cell proliferation, while no responses against ovalbumin (OVA) or lysozyme were observed. Values are the mean and SEM of triplicate experiments. Non-Tx = nonthymectomized. B, Flow cytometric analysis, showing that the activation markers $CD44^{high}$, $CD45RB^{low}$, and $Mel\text{-}14^{low}$ 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.