Autoimmunity against a Tissue Kallikrein in IQI/Jic Mice

A MODEL FOR SJÖGREN’S SYNDROME*

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We have recently characterized IQI/Jic mice as a model for Sjögren’s syndrome (SS), a chronic autoimmune disease in humans. In SS, local lymphocytic infiltrations into salivary and lacrimal glands frequently develop to the involvement of systemic exocrine and nonexocrine organs, and the mechanism for progression of this disease remains obscure. Herein, we report identification of an autoantigen shared by various target organs in IQI/Jic mice. Polypeptides identified based on immunorecognition by autoantibodies in sera from IQI/Jic mice affected with autoimmune disease (>12 weeks of age) were tissue kallikrein (Klk)-1 and -13 and were cross-reactive to the autoantibodies. Interestingly, Klk-13, but not Klk-1, caused a proliferative response of splenic T cells from IQI/Jic mice from the age of 4 weeks onward. In addition, remarkably enhanced expression of Klk-13 was observed in the salivary glands of the mice in accordance with the development of inflammatory lesions. These results indicate that Klk-13 acts as an autoantigen and may increase T cells responsive to organs commonly expressing Klk-13, playing a pivotal role in the etiology of progression of disease in IQI/Jic mice. Our findings provide insights into the contributions of autoantigens shared by multiple organs in the progress of SS from an organ-specific to a systemic disorder.

Sjögren’s syndrome (SS)† is a chronic autoimmune disease characterized by lymphocytic infiltration and tissue damage to the salivary and lacrimal glands, which results in dryness of the mouth (xerostomia) and eyes (keratoconjunctivitis sicca) (1, 2). Patients with SS often show a wide spectrum of autoimmune-related disorders, including pancreatitis, sclerosing cholangitis, interstitial nephritis, and interstitial pneumonitis. In these target organs, different epithelial cells in exocrine and nonexocrine tissues are primarily affected, and SS has been generally referred to as “autoimmune exocrinopathy,” “dry gland syndrome,” or, more recently as “autoimmune epitheliitis” (3–5). These clinical observations have led to the assumption that autoimmune reactions against antigens commonly expressed in epithelial cells play a pathogenic role in this disease (6). Characterization of novel autoantigens associated with the systemic involvement in SS would provide useful information to better understand its pathogenesis and to develop new diagnostic and therapeutic strategies.

Previous efforts have detected circulating antibodies and/or T cells reactive with various autoantigens in SS, including several nuclear complexes (SS-A/Ro, SS-B/La, SS-56, etc.) (7), the type 3 muscarinic acetylcholine receptor (8), a cleavage fragment of α-fodrin produced during the apoptosis of salivary epithelial cells (9, 10), and islet cell autoantigen 69 (11). However, the molecular nature of autoantigens causing the autoimmunity against the different epithelial cells in multiple organs in SS has remained obscure.

Several animal models have been used to investigate the pathogenesis of SS. They include autoimmune-prone mice that develop SS-like pathology associated with other autoimmune conditions such as systemic lupus erythematosus, rheumatic arthritis, and insulin (12–15) and other rodent strains requiring experimental manipulations such as antigen sensitization and neonatal thymectomy to develop inflammatory lesions (6, 16, 17). Of these, IQI/Jic mice are the most recently identified (18, 19). They spontaneously develop autoimmune infiltration of lymphocytes in the salivary gland at the age of 8 weeks, thereafter progressing to systemic disorder with the involvement of the lacrimal gland, pancreas, kidney, and lung (20, 21). Based on this characteristic unique to IQI/Jic mice, we previously suggested them to be favorable for studies on the pathogenesis of autoimmune epitheilitis in SS and hypothesized that there are autoimmune reactions against antigens shared by target organs in IQI/Jic mice. Although the inflammatory lesions contain a large number of B cells suggestive of a progressive humoral immune response, serum autoantibodies against salivary tissues, one of the prominent pathophysiological features in patients with SS and animal models (7, 22–25), have not been detected by immunohistochemistry in IQI/Jic mice (18).

In this study, we first demonstrated the presence of IgG autoantibodies characteristic to IQI/Jic mice by immunoblotting and identified the targets of these autoantibodies as kallikrein (Klk)-1 and -13, which are known to be localized mainly in glandular tissues (26). Then we examined expression of and T cell proliferative responses to Klk-1 and -13 to investigate roles of these molecules in autoimmune pathogenesis in IQI/Jic mice.

EXPERIMENTAL PROCEDURES

Animals—IQI/Jic mice were bred from breeding pairs purchased from Crea Japan (Tokyo, Japan) and maintained in our own animal facility. Animals were housed in a barrier facility and maintained in a temperature- and humidity-controlled environment on a 12-h light/dark cycle. The animal care and use were performed in accordance with the institutional guidelines.

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¶The abbreviations used are: SS, Sjögren’s syndrome; Klk, kallikrein; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride.
facility. Female IQI/Jic mice of various ages were utilized for this study. BALB/c and C57BL/6 mice purchased from Japan SLC (Shizuoka, Japan) were used as sex- and age-matched controls. The animals were maintained under specific pathogen-free conditions. All of the experimental procedures met with the approval of Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University.

**Immunoblotting**—Various tissues from 16-week-old IQI/Jic mice were homogenized with a Polytron mixer (Kinematica, Lucerne, Switzerland) in 20 mM Tris- HCl (pH 7.2), 100 mM NaCl, and 1% Triton X-100. After centrifugation at 105,000 g for 30 min, the proteins in the supernatant were treated with 3% β-mercaptoethanol (Wako, Osaka, Japan) at 95 °C for 3 min, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were incubated with sera (diluted 1:100) from different strains, followed by the reaction with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad) and visualization with the ECL system (Amersham Biosciences). Salivary extracts of IQI/Jic mice of various ages and BALB/c and C57BL/6 mice were also prepared and subjected to immunoblot analysis with sera from IQI/Jic mice.

**Purification and Identification of the Salivary Antigens**—Detergent-free extracts of the salivary glands from IQI/Jic mice at the ages of 16–24 weeks were prepared using a Polytron mixer in 50 mM Tris-HCl buffer (pH 5.0) and 100 mM NaCl, and 1% Triton X-100. After centrifugation at 105,000 g for 30 min, the proteins in the supernatant were treated with 3% β-mercaptoethanol (Wako, Osaka, Japan) at 95 °C for 3 min, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were incubated with sera (diluted 1:100) from different strains, followed by the reaction with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad) and visualization with the ECL system (Amersham Biosciences). Salivary extracts of IQI/Jic mice of various ages and BALB/c and C57BL/6 mice were also prepared and subjected to immunoblot analysis with sera from IQI/Jic mice.

**Reverse Transcription-PCR**—Total RNA was extracted from various organs of 16-week-old IQI/Jic mice with an RNA extraction reagent (Invitrogen). The cDNA synthesis was performed using a reverse transcription kit (Invitrogen), according to the manufacturer’s directions. The cDNA was amplified by PCR with the following primers: 5′-AGAGCTCCACGACATA-3′ and 5′-GAATTCATCATTTCCAGGATT-3′, indicating 18 and 16-kDa antigens, respectively. Extracted proteins in phosphate-NaOH buffer were separated on a Q Sepharose FF column (Amersham Biosciences) with a linear gradient of 0–0.5 M NaCl in the same buffer. The extract in Tris-HCl buffer was similarly fractionated using an S Seprhase FF (Amersham Biosciences) column and then a Q Sepharose FF column. Antigen-containing fractions from each sample were identified by immunoblotting with sera of IQI/Jic mice and were separately fractionated on a Superdex 75HR column (Amersham Biosciences). Antigen-containing fractions were subjected to further purifications by reversed phase high performance liquid chromatography using an RPC resource column (Amersham Biosciences). Proteins in the positive fractions indicated by arrows in Fig. 2B were separated on SDS gels and transferred to nitrocellulose filters. Autoreactive polypeptides were recognized even in immunoblotting without serum (Fig. 1C), whereas sera from age-matched control mice did not react with heavy and light chains of IgG in tissue extracts, because they were recognized even in immunoblotting without serum (Fig. 1B). Notably, sera from IQI/Jic mice reacted with 18- and 16-kDa polypeptides in salivary gland extracts with high intensities, whereas sera from age-matched control mice did not show such signals in immunoblotting (Fig. 1A), indicating that IQI/Jic mice had autoantibodies to these polypeptides relatively characteristic of the salivary gland, a primary target organ in their autoimmune disease. These autoantibodies appeared in sera of IQI/Jic mice from 12 weeks of age onward; autoantibodies were detected in three of six mice examined at the age of 12 weeks and in all mice at the ages of 16, 32, and 48 weeks (six of six for each age group). In addition, signal intensities in immunoblotting increased as the mice aged when tissue extracts from 16-week-old IQI/Jic mice were used for antigens (data not shown). Although sera from several IQI/Jic mice showed faint bands of some other polypeptides in nontarget organs, including the brain, liver, and/or stomach (Fig. 1A), the pathologic significance of these signals was obscure, and they were excluded from further examination in this study.

**Identification of the 18- and 16-kDa Salivary Antigens**—To identify the autoantigens, the 18- and 16-kDa polypeptides in the salivary glands of IQI/Jic mice were purified by a series of chromatographies based on recognition with autoantibodies in immunoblotting. After a sequential procedure employing anion and cation exchange chromatographies followed by gel permeation, the 18- and 16-kDa polypeptides were separated to ho-
mogeneity on a reversed phase chromatography column (Fig. 1, B and C). Sequencing analysis gave a single N-terminal amino acid sequence for each 18- and 16-kDa polypeptide purified. The N-terminal sequence of the 18-kDa polypeptide consisting of 18 amino acid residues was identical to that of the heavy chain of murine kallikrein (Klk)-1/glandular Klk-6 (28) except for two unidentified residues, the seventh and fourteenth residues, as shown in Fig. 1D. Likewise, the 16-kDa polypeptide

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**Fig. 1. Presence of circulating autoantibodies in IQI/Jic mice and identification of antigens.** A, extracts of various tissues from IQI/Jic mice at the age of 16 weeks were subjected to SDS-PAGE, followed by the staining with Coomassie Brilliant Blue (CBB) or immunoblot analyses with or without sera from 16-week-old IQI/Jic, BALB/c, and C57BL/6 mice. The arrows in the magnified blot indicate the 18- and 16-kDa antigens in salivary gland extracts specifically reactive with sera from IQI/Jic mice. B, chromatograms of reverse phase high performance liquid chromatography as the final step for purification of 18-kDa (left) and 16-kDa (right) antigens. The solid and dotted lines indicate the absorbance at 280 nm and the concentration of acetonitrile in eluting buffer, respectively. C, peaks marked with arrows in the chromatograms (B) were immunoreactive with sera from IQI/Jic mice. D, comparison of the N-terminal amino acid sequences of the purified 18- and 16-kDa antigens with those of murine Klk-1 and -13, respectively. The initial and repetitive yields of amino acids were 46.8 and 2.1 pmol for the 18-kDa antigen, and 19.0 and 0.3 pmol for the 16-kDa antigen, respectively. X, unidentified residues.
was identified as Klk-13/glandular Klk-13 (28) heavy chain (Fig. 1D). Here the first 20 amino acid residues were identical to those of Klk-13, again except for two unidentified residues. These Klks are known to be heterodimeric molecules consisting of heavy and light chains, and the primary structures of heavy chains are highly homologous (28, 29) as represented by the N-terminal sequences shown in Fig. 1D.

Expression Profiling of Klk-1 and -13—Immunoblotting analysis also showed that the 18- and 16-kDa antigens in the salivary glands of IQI/Jic mice became abundant as the mice aged, that is, these polypeptides were clearly observed in salivary gland extracts from mice older than 16 weeks but not in those from young animals at the age of 4 weeks (Fig. 2A). These observations coincided with our previous findings that inflammatory infiltrations in the salivary glands were apparent in old mice (>16 weeks) but not in young mice (21), suggesting that the production of autoantibodies and the apparent increase of the antigens were associated with the development of the disease in IQI/Jic mice. The absence or the very low intensities of 18- and 16-kDa polypeptides in tissues from BALB/c and C57BL/6 mice (Fig. 2A) raised the possibility that the contents of these antigens were extremely low or that antigenicities of these polypeptides in control mice were different from those in IQI/Jic mice. Moreover, if Klk-1 and/or Klk-13 are associated with the disease development as autoantigens, their expression in various tissues could be expected, because IQI/Jic mice spontaneously develop inflammatory lesions in the lacrimal gland, lung, pancreas, and kidney in addition to the salivary gland (21), although no bands were visualized on immunoblots of extracts from these tissues (Fig. 1A). Therefore, we then examined the expression of Klk-1 and -13 in these target organs from IQI/Jic mice by reverse transcription-PCR and found that the transcripts of these Klks were present in all of these tissues (Fig. 2B). Furthermore, quantitative PCR analyses demonstrated the abundance of Klk-1 and -13 transcripts in the salivary gland at levels clearly distinguishable from those in other organs (Fig. 2C). In addition, cloning and sequencing of Klk-1 and -13 cDNAs of various organs from IQI/Jic mice demonstrated that the deduced amino acid sequences of these Klks were identical to those reported for ICR (28) and BALB/c (30) mice,2 negating the possibility of structural differences for Klks among strains as the cause of the absence or low intensities of 18- and 16-kDa bands in tissues from control mice on the immunoblot (Fig. 2A).

Cross-reactivity of Serum Autoantibodies with Reduced Klks—SDS-PAGE under reduced and nonreduced conditions showed that purified Klk-1 and -13 were heterodimeric molecules in which heavy and light chains were linked via disulfide bonds (Fig. 3A). Immunoblot analysis revealed that autoantibodies found in IQI/Jic mice were clearly reactive with the 18-kDa (Klk-1) and 16-kDa (Klk-13) heavy chains of reduced forms but not with native Klks or the light chains of the reduced forms (Fig. 3A). This was verified by the observation that the recombinant glutathione S-transferase-fused protein of Klk-1 heavy chain specifically reacted with sera from IQI/Jic mice, whereas the light chain recombinant protein showed no positive reactions (Fig. 3B). Because heavy chains of murine Klk-1 and -13 are known to be highly homologous in their primary structures (28, 29), we then examined the cross-reactivity of autoantibodies in sera of IQI/Jic mice with these antigens. Immunoblot analysis showed that autoantibodies once bound to Klk-1 and -13 reacted with Klk-13 and Klk-1, respectively (Fig. 3C).

Cellular Autoreactivity with Klk-13 in IQI/Jic Mice—Splenocytes from IQI/Jic mice were tested for proliferation in response to purified Klks to investigate the reactivity of T cells with these molecules. As shown in Fig. 4A, spleen cells from IQI/Jic mice of various ages showed significant proliferative responses to native Klk-1 and -13 but not to the control antigen, bovine serum albumin. The proliferative response to Klk-13 was specifically observed in IQI/Jic mice.
contrast, the response to Klk-1 was detected in normal BALB/c and C57BL/6 as well. It has been demonstrated that Klk-1 enhances the spontaneous proliferation of lymphocytes through its enzymatic activity of serine protease (27). Treatment of antigens with a serine protease inhibitor, AEBSF, completely abolished the proliferative response to Klk-1 of splenocytes not only from control mice but also from IQI/Jic mice (Fig. 4B). However, the significant response to Klk-13 in IQI/Jic mice was evident even after the treatment with AEBSF (Fig. 4B). Fig. 4B also shows that T cell autoreactivity with Klk-13 appeared spontaneously in IQI/Jic mice at the age of 4 weeks, much earlier than the occurrence of inflammatory lesions and production of serum autoantibodies and was maintained at a constant level afterward. Moreover, proliferation of T cells from IQI/Jic mice was elicited in response to Klk-13 in a dose-dependent manner but not in response to Klk-1 over a wide range of concentrations from 0.05 to 50 μg/ml (Fig. 4C).

**DISCUSSION**

We previously reported that the IQI/Jic strain of mice is a unique model for SS, because it represents the autoimmune epithelitis process, progressing from salivary-specific disorders to the involvement of multiple organs with inflammatory cellular infiltrations (21). In the present study, we demonstrated the presence of autoantibodies reactive with the extract of salivary glands, the primary target of autoimmunity, in the sera of IQI/Jic mice (Fig. 1A) and identified the antigens as kallikrein (Klk)-1 and -13 (Fig. 1, B–D). Autoantibodies in sera of IQI/Jic mice were cross-reactive with these Klks (Fig. 3C), and T cell proliferation occurred specifically in response to Klk-13, regardless of the treatment with a serine protease inhibitor (Fig. 4). Based on these results, we concluded that IQI/Jic mice bear T cell autoimmunity against Klk-13, and the resulting autoantibodies are reactive with Klk-1 as well, presumably because of high homology in the amino acid sequences of these molecules (28, 29).

Transcripts of Klk-13 were detected commonly in various target organs (Fig. 2B), and their expression levels were significantly higher in the salivary gland than in other organs in IQI/Jic mice (Fig. 2C). Klk-13, formerly designated prorenin converting enzyme B (31) or epidermal growth factor-binding protein B (32), has been considered to be expressed most abundantly in the duct epithelial cells in salivary glands (33, 34), where the lesions of lymphocytic infiltrations initially appear in IQI/Jic mice (21). Accordingly, autoimmune reaction against Klk-13 could play a critical role in disease progression from periductal infiltrations within salivary glands to systemic autoimmune conditions targeting multiple organs in IQI/Jic mice. To ensure the correlation of autoimmunity against Klk-13 with the development or spread of the disease in IQI/Jic mice, future
studies should include in vivo analyses of the adoptive transfer of Klk-13-reactive T cells (35, 36) and the induction of Klk-13-specific tolerance to IQI/Jic mice by neonatal (9) or intrathymic (37) injection of Klk-13.

Klks are a group of serine proteases generally classified into two major categories based on their localization: one in blood plasma called plasma Klk and another secreted mainly by glandular organs called tissue Klk (26). Their general function is to convert inactive kininogen to active kinins, which have been considered to regulate the local blood pressure, blood flow, and electrolyte balance (33). Additionally, accumulating evidence indicates that the kallikrein cascade plays a significant role in the initiation and maintenance of inflammatory responses (38, 39). In this study, significant increases of Klk-1 and -13, both tissue Klks, were observed in salivary glands from IQI/Jic mice at the age of 16 weeks, subsequently being at an equivalent level afterward up to 48 weeks of age (Fig. 2A). We previously reported that, in IQI/Jic mice, minute sialoadenitis could be detected at the age of 8 weeks, and focal infiltration of mononuclear cells became apparent after 16 weeks of age, progressing to severe parenchymal destruction as they aged (20, 21). These observations indicate that up-regulation of Klks in salivary glands involves the early development of sialoadenitis in IQI/Jic mice. In humans, saliva from patients with SS has been reported to contain significantly increased levels of Klks considered to be derived from affected duct epithelial cells (40, 41). However, similar to our findings in IQI/Jic mice, no correlation has been found between the Klk levels in saliva and the severity of disease, including the amount of saliva production and the histological degree of inflammatory lesions in the affected tissues in these patients (40). Collectively, increased Klks could result from the early inflammation in salivary glands to form a basis for the progression or persistence of sialoadenitis in IQI/Jic mice. Furthermore, release of an increased amount of Klk-13 from the salivary glands infiltrated by antigen-presenting cells could abrogate the tolerance to this molecule and accelerate the immune reaction, acting as an autoantigen.

Detailed immunoblot analysis revealed that the anti-Klk antibody in the sera of IQI/Jic mice binds to the autoantigens under the reduced condition but not to their native forms (Fig. 3A). This fully explains the negative results of a previous report (18) and our preliminary studies in which anti-salivary autoantibodies were not detected in sera of the mice by immunohistochemistry. Serum autoantibodies reactive with salivary

![Fig. 4. Examination of cellular autoimmunity to Klks in IQI/Jic mice. A, splenocytes from IQI/Jic at various ages (4–32 weeks) and 16-week-old BALB/c and C57BL/6 mice (n = 4–6/group) were examined for proliferative responses to bovine serum albumin (BSA, 10 μg/ml, control antigen), purified Klk-1 (10 μg/ml), and Klk-13 (10 μg/ml). The Klk-13 induced proliferative response was only in IQI/Jic mice, but the response to Klk-1 was seen in control mice as well. B, proliferative responses of splenocytes to bovine serum albumin, Klk-1, and Klk-13 treated with AEBSF, a serine protease inhibitor. The response to Klk-1 was completely abolished after the inhibition of serine protease activity in all groups, whereas the response to Klk-13 in IQI/Jic mice was marked. C, proliferation of splenocytes from IQI/Jic mice at 8–16 weeks of age in response to AEBSF-treated Klks at different concentrations (0.05–50 μg/ml). A dose-dependent response was elicited to Klk-13 but not to Klk-1. *p < 0.05; **p < 0.01 for A–C.](http://www.jbc.org/)

epithelial cells have been detected in patients with SS by immunohistochemical antibodies in SS of 15–78% (22–25). Our serological evaluation in IQLi/Jic mice suggests a limitation of this conventional method, which could have failed to detect putative autoantibodies generated against the epitopes hidden within the native dimeric molecules. In addition, various autoantibodies have been shown to directly relate to the pathogenesis of autoimmune diseases by recognizing native antigens and hampering their physiological functions (42–44). In patients with SS (8) and a murine model (45), glandular secretions are impaired by autoantibodies directed against autonomic nervous system receptors, including muscarinic receptors, on the cell surface of lacrimal and salivary glands. However, the autoantibodies in IQLi/Jic mice are unlikely to cause the alteration of enzymatic activity of Kiks to affect local inflammatory responses because of the lack of association between the autoantibody and native Kiks.

In conclusion, we identified Klk-13 as an autoantigen in the IQLi/Jic mouse model for SS. Overexpression of Klk-13 in the early phase of sialoadenitis is believed to increase autoreactive T cells capable of generating an autoimmune response against multiple organs commonly expressing Klk-13. Our results provide insights into the role of autoantigens in the disease process from organ-specific to systemic disorder in SS.

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