of Sjögren's syndrome, but not from Fas-deficient MRL/lpr mice and FasL-deficient MRL/gld mice. FasL undergoes metalloproteinase (MMP)-mediated proteolytic processing in its extracellular domains, resulting in the release of soluble trimeric ligands (soluble FasL, sFasL). In this case, the processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells, and a significant increase in expressions of MMP-9 mRNA was observed in spleen cells from the mouse model of Sjögren's syndrome[46]. *In vitro* T cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in the murine SS model. The data indicate that the increased generation of soluble FasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells[46]. Our study demonstrates that an organ-specific autoantigen may play an important role in the down-modulation of FasL-mediated AICD. The increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells in the murine SS model. Previous studies have demonstrated that $CD4^+$ T cells are susceptible to AICD induced through TCR-mediated recognition of allogeneic MHC class II molecules, supporting the notion that AICD can be triggered in activated T cells through the TCR-mediated recognition of antigen[47-49]. Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders[7,50]. In proteoglycan-induced arthritis, $CD4^+$ T cells proliferate at a high rate in response to proteoglycan stimulation, and exhibit a Th1-type response[51]. The study investigated whether when Th1 cells were dominant, disease outcome could be modified with pharmacological amounts of Th2 cytokines. Treatment with IL-4 prevented disease and induced a switch from a Th1-type to a Th2-type response. Proinflammatory cytokine mRNA transcripts were reduced in joints of cytokine-treated mice. Th2 cytokine therapy at the time of maximum joint inflammation also suppressed symptoms of disease. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of Sjögren's syndrome. $CD4^+$ T cells are susceptible to AICD induced through TCR-mediated recognition of allogeneic MHC class II molecules[52,53]. Our data demonstrated that autoantigen stimulation results in a significant decrease in anti-Fas-induced $CD4^+$ T cell apoptosis in a dose-dependent manner. In addition, AICD is triggered in $CD4^+$ T cells by a specific antigenic peptide, e.g. tetanus toxoid or myelin basic protein, presented by the appropriate MHC class II molecules (54), supporting the notion that AICD can be triggered in activated cells through the TCR-mediated recognition of antigen. The specificity of cytotoxic T lymphocyte (CTL) function has been an important issue of organ-specific autoimmune response, but little is known about the events triggering T cell invasion of the target organs as a prelude to organ-specific autoimmune diseases.

Tissue-specific apoptosis induced by estrogen deficiency

Recently, we have evaluated the effects on autoantigen cleavage in estrogen deficient healthy C57BL/6 (B6) mice treated with an ovariectomy (Ovx) (Fig. 3A). We have demonstrated a significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen deficient healthy C56BL/6 (B6) mice[55] (Fig. 3B). These data suggest that α-fodrin cleavage triggered by estrogen deficiency plays a role in the development of autoimmune exocrinopathy in the salivary and lacrimal glands. In contrast, apoptotic cells in the salivary glands were not found in ERαKO mice. In *in vitro* studies using primary cultured mouse salivary gland cells (MSG) and human salivary gland cells (HSG), we found a cleavage product of 120 kDa α-fodrin in cells that had undergone tamoxifen (Tam)-induced apoptosis, but not in other types of cells including MCF-7. Since pretreatment with estrogen inhibits the Tam-induced apoptosis of MSG and HSG cells, estrogen may play a crucial role in the apoptosis-related signal pathway. When we analyzed whether cystein proteases are involved in Tam-induced apoptosis of HSG cells, we observed a time-dependent increase in the active forms of caspase 1. In addition, we found that the promoter activity of caspase 1 was significantly increased when HSG cells transfected with the promoter-caspase 1 gene were stimulated with Tam. Indeed, active forms of caspase 1 were detected only in the salivary gland tissues from Ovx-B6 mice. Of importance is that adoptive transfer of α-fodrin-reactive T cells into Ovx-B6 mice resulted in the development of autoimmune exocrinopathy similar to SS. It has been strongly suggested that α-fodrin

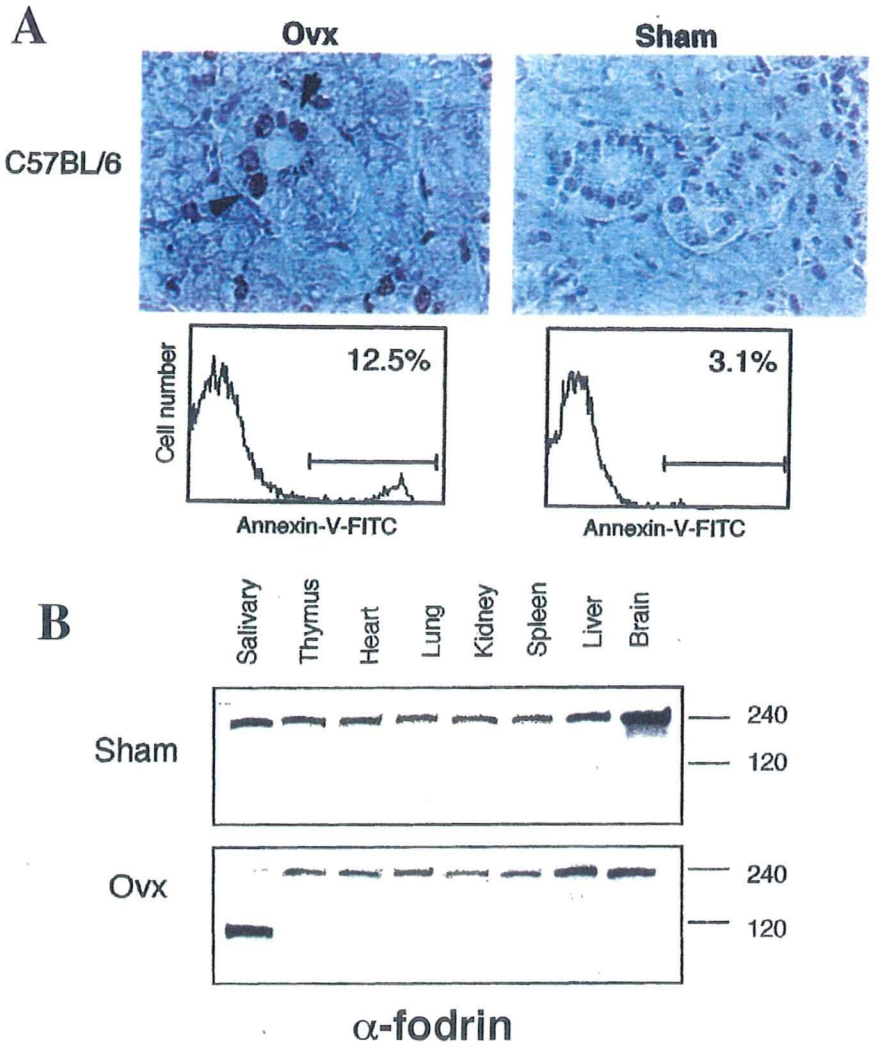

Fig. 3 Tissue-specific apoptosis in the salivary glands induced by estrogen deficiency[55]. (A) Detection of TUNEL$^+$-apoptotic cells in the salivary gland sections from Ovx- and Sham-C57BL/6 (B6) mice at 3 weeks after Ovx (arrows). Annexin V-flow cytometric analysis using MSG cells demonstrated 12.5%-positive cells detected in Ovx-B6 mice, but 3.1%-positive in Sham-B6 mice. Data are represented in triplicate. (B) Detection of α-fodrin cleavage in various tissues from Ovx- and Sham-B6 mice on Western blot analysis showing the distinct band of 120 kDa in the salivary gland tissue alone from Ovx-B6 mice.

fragments induced by estrogen deficiency may play an important role in the development of autoimmune lesions in Sjögren's syndrome[55]. We have demonstrated that antiestrogenic actions, including estrogen deficiency, may have a crucial influence on apoptosis and α-fodrin proteolysis through an increased caspase activity in the salivary gland cells, suggesting a novel mechanism for the development of organ-specific autoimmunity in postmenopausal women. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are now being investigated.

Apoptosis in labial gland biopsies with Sjögren's syndrome

To evaluate tissue-specific apoptosis and autoantigen cleavage in the salivary glands with Sjögren's syndrome, we analyzed human biopsy specimens by immunohistology and immunoblotting. All patients with SS were female, had documented xerostomia and

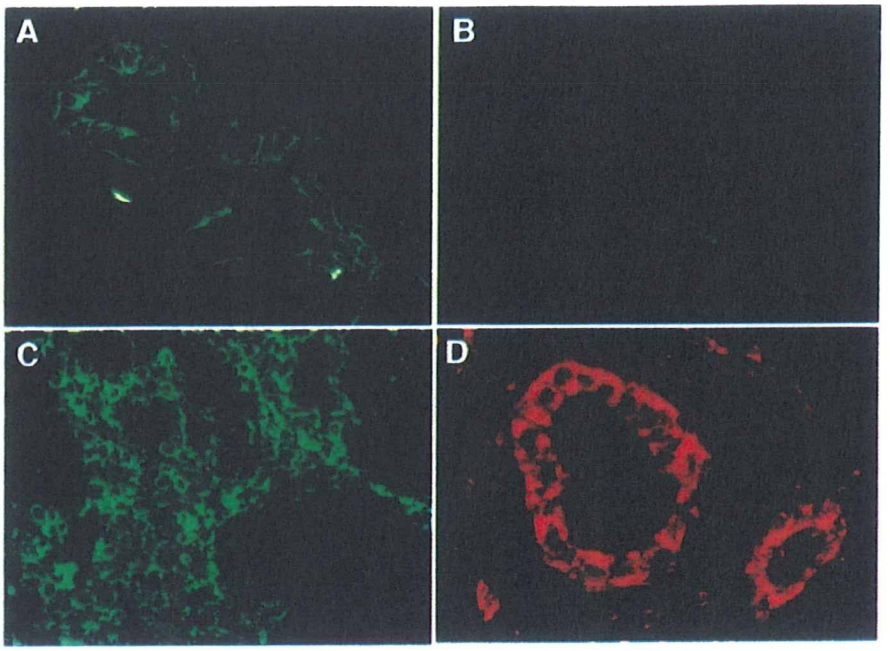

Fig. 4 Immunohistological features in the labial gland biopsies with Sjögren's syndrome. (A) A cleavage product of 120 kDa α-fodrin was present exclusively in epithelial duct cells of the SS salivary glands with Sjögren's syndrome, but not in (B) control salivary glands with mucous cyst. (C) Immunofluorescence analysis revealed that the majority of tissue-infiltrating lymphoid cells bore FasL, and (D) epithelial duct cells stained positively with Fas on their cell surface in SS salivary glands with Sjögren's syndrome. Six samples for each were examined.

keratoconjunctivitis sicca, and fulfilled the San Diego criteria for the diagnosis of Sjögren's syndrome. Analyses were performed under the certification of the ethics board of Tokushima University Hospital. Immunofluorescence analysis using polyclonal Ab against synthetic 120 kDa α-fodrin demonstrated that a cleavage product of α-fodrin was present in epithelial duct cells of the labial salivary gland biopsies from SS patients, but not in control glands (Fig. 4A & 4B). Western blot analysis confirmed the same results, indicating that a cleavage product of 120 kDa α-fodrin is present in the diseased glands with Sjögren's syndrome. Immunohistochemical analysis revealed that a majority of infiltrating cells were $CD4^+$, and that a small number of $CD8^+$ cells were present in the salivary glands with Sjögren's syndrome. Immunofluorescence analysis revealed that a large number of infiltrating lymphoid cells bear FasL in the salivary glands with Sjögen's syndrome (Fig. 4C), and epithelial duct cells stained positively with Fas on their cell surface (Fig. 4D). Thus, we provided evidence suggesting that Fas/FasL-mediated apoptosis may be involved in *in vivo* cleavage of α-fodrin autoantigen in the salivary glands with Sjögren's syndrome.

On the horizon

A cleavage product of 120 kDa α-fodrin was identified as an important organ-specific autoantigen in human Sjögren's syndrome. The data discussed in this review are strongly suggestive of the essential role of caspase cascade for α-fodrin autoantigen cleavage leading to tissue destruction in autoimmune exocrinopathy in Sjögren's syndrome. Alpha-fodrin cleavage by caspases can potentially lead to cytoskeletal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain[56]. It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles[57], suggesting that a cleavage product of 120 kDa α-fodrin could be a novel component of unknown immunoregulatory networks such as cytolinker proteins[58]. *In vitro* T cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells

from murine Sjögren's syndrome. The processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells *in vivo*, and a significant increase in expressions of MMP-9 mRNA was observed in spleen cells from the mouse model. These data indicate that the increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells. We have recently observed that $CD4^+$ T cells freshly isolated from PBMC with SS patients induce Th1 cytokine (IL-2, IFN-γ), suggesting that the autoantigen peptide plays an important role in the Th1/Th2 balance *in vivo*. Moreover, α-fodrin peptide-pulsed $CD4^+$ T cells down-regulate Fas-mediated apoptosis when pulsed with each corresponding peptide. Although antigen-induced T cell death is known to be regulated by CD4 expression, molecular mechanisms responsible for T cell death should be further elucidated. However, it remains unclear whether T cells specific for endogenous epitopes play a significant pathologic role in tissue damage during clinical episodes. Our data demonstrated that AFN peptide-stimulation results in a significant decrease in anti-Fas-induced $CD4^+$ T cell apoptosis. 120 kDa α-fodrin, the apoptosis associated breakdown product, may play an important role in the development of SS, and that the autoantigen peptide is a novel participant in the down-modulation of Th1/Th2 balance and peripheral T cell tolerance. It is a future possibility that a peptide analogue of autoantigen could be used as an immunotherapeutic agent.

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REVIEW ARTICLE

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Revised Japanese criteria for Sjögren's syndrome (1999): availability and validity

Abstract The Japanese criteria for diagnosing Sjögren's syndrome (SS) were revised in 1999, and consist of four major areas: histopathology, oral examination, ocular examination, and serological examination. A diagnosis of SS can be made when the patient meets at least two of these four criteria. This report describes how the revised Japanese criteria were established. After the publication of the revised Japanese criteria (1999), a research study which focused on evaluating its availability and validity was carried out in 2001 using funds from Grant-in-Aids for Scientific Research supported by the Japan Society for the Promotion of Science. The availability of the revised criteria was investigated by a questionnaire study through the Japanese Medical Society for Sjögren's Syndrome, and the use of the revised criteria for diagnosing SS in these medical facilities was found to be 76%. To evaluate the validity of the revised criteria, the records of 900 patients, including SS patients and non-SS controls, from 54 clinical centers were registered and analyzed to calculate the accuracy of the criteria. The revised Japanese criteria were found to have 96.0% sensitivity, 90.5% specificity, and 94.5% accuracy for diagnosing SS.

Key words Diagnostic criteria · Revised Japanese criteria · Sensitivity · Sjögren's syndrome (SS) · Specificity

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Introduction

The Japanese diagnostic criteria for Sjögren's syndrome (SS)[1] were revised in 1999, as shown in Table 1. The previous Japanese criteria had been established in 1977 and published in 1978 in a report entitled *Research Committee on Sjögren's Syndrome of the Japanese Ministry of Health and Welfare.*[2] These old criteria were widely used for 20 years and made a great contribution to the clinical diagnosis of SS in Japan. However, the developments in scientific research and diagnostic technology with regard to SS then led to a revision of the diagnostic criteria. The Research Committee on Autoimmune Diseases of the Japanese Ministry of Health and Welfare requested the cooperation of the Japanese Medical Society for Sjögren's Syndrome in revising the criteria. In 1994, the Society established a board as a working group to revise the criteria. This worked for over 5 years, and reported its progress at every annual meeting of the Society. The final revision was completed at the annual meeting in 1998. The final report was then submitted to the national committee, and published as the revised Japanese criteria for SS in 1999.

After issuing the revised criteria, we planned to assess their availability and validity. This assessment was performed in 2001 using funds from Grant-in-Aids for Scientific Research supported by the Japan Society for the Promotion of Science. This article gives a brief history of how the revised criteria were achieved, and the results of the investigative assessment into their availability and validity.

Table 1. Revised Japanese Criteria for Sjögren's Syndrome (SS) (1999)

1. Histopathology
   Definition: Positive for at least one of (A) or (B):
   A) Focus score ≧ 1 (periductal lymphoid cell infiltration ≧ 50) in a 4-mm^2 minor salivary gland biopsy
   B) Focus score ≧ 1 (periductal lymphoid cell infiltration ≧ 50) in a 4-mm^2 lacrimal gland biopsy
2. Oral Examination
   Definition: Positive for at least one of (A) or (B):
   A) Abnormal findings in sialography ≧ Stage I (diffuse punctate shadows of less than 1 mm)
   B) Decreased salivary secretion (flow rate ≦ 10 ml/10 min according to the chewing gum test or ≦ 2 g/2 min according to the Saxon test) and decreased salivary function according to salivary gland scintigraphy
3. Ocular Examination
   Definition: Positive for at least one of (A) or (B):
   A) Schirmer's test ≦ 5 mm/5 min and rose bengal test ≧ 3 according to the van Bijsterveld score
   B) Schirmer's test ≦ 5 mm/5 min and positive fluorescein staining test
4. Serological Examinatison
   Definition: Positive for at least one of (A) or (B):
   A) Anti-Ro/SS-A antibody
   B) Anti-La/SS-B antibody

Diagnostic criteria: Diagnosis of SS can be made when the patient meets at least two of the above four criteria

Materials and methods

Before revision

In 1994, the Japanese Medical Society for Sjögren's Syndrome established a board as a working group for the revision of the old criteria. The board consisted of seven members, Takashi Fujibayashi, Susumu Sugai, Nobuyuki Miyasaka, Takeshi Tojo, Shoji Miyawaki, Yukinobu Ichikawa, and Kazuo Tsubota, and each belonged to a different medical clinical center. The subspecialities of the members were internal medicine, especially rheumatology (S.S., N.M., T.T., S.M., and Y.I.), ophthalmology (K.T.), and oral medicine (T.F.). During the first meeting of the board, which was held in Tokyo on September 20, 1994, the general policy and procedures of the revision were discussed. An understanding was reached with regard to the following aims: (1) to make a new revised criteria based on the old Japanese criteria; (2) the results to be judged academically by objective examinations; (3) to be reasonable in consideration of international standards; (4) a subjective feeling of dryness should not always be a necessary prerequisite condition; (5) to make only definite diagnoses and avoid probable cases; (6) to consider new diagnostic techniques such as salivary gland scintigraphy; (7) to consider objective examinations such as serum autoantibodies; (8) to revise the histopathological scale from one focus per lobe of the minor glands, as used under the old Japanese criteria, to one focus per 4 mm^2, as widely used by other countries; (9) to consider whether primary and secondary SS require different rules based on the results of the study. However, after consultation, it was agreed that it was desirable that these could be diagnosed using identical standards. At the second meeting, in November 1994, the survey protocol form used to register each patient's record was discussed, and a form with 42 items, including the necessary examination records for diagnosing SS, was agreed upon. The seven members of the board were asked to register 60 consecutively applicable patients' records from their own files. These were to be grouped as follows: 15 patients with primary SS, 15 with secondary SS, 15 control patients (e.g., with simple dry mouth or dry eye but without SS) as a control for primary SS, and 15 patients with connective tissue diseases (CTD) but without SS as a control for secondary SS. It was agreed that the preliminary selection of patients with or without SS should be made using each board member's clinical judgment. A total of 419 cases had been registered by July 1995. These patients included 129 cases of primary SS, 106 of secondary SS, 108 primary SS controls, and 76 secondary SS controls. The data analysis of these patient's records was carried out at the office of the board (Department of Oral and Maxillofacial Surgery, Dokkyo University School of Medicine, where T. Fujibayashi was then working), and the results were distributed to the board members. Each board member was asked to create and submit at least one draft plan of revised diagnostic criteria. The sensitivity, specificity, and accuracy of every draft plan, as well as those of each examination used in diagnosing SS, were assessed for the 419 cases registered at the office. After several revisions of each draft plan and discussions at several board meetings, three tentative preliminary plans for the revised criteria were submitted to the 5th general meeting of the Japanese Medical Society for Sjögren's Syndrome in Sapporo in September 1995. Preliminary revised criteria were submitted to the national committee in 1996.[3] However, an additional large-scale study was recommended at the 6th general meeting in Nagoya in 1996. At that time, an additional 404 patients' records were registered from 22 clinical centers countrywide, from other members of the society who had agreed to cooperate in this investigation. Each unit was requested to submit 20 consecutively applicable patients' records from their own files, grouped as follows: 5 patients with primary SS, 5 patients with secondary SS, 5 patients as controls for primary SS, and 5 patients with CTD but without SS as controls for secondary SS. In total, 823 cases (419 from the board

members and 404 from the Society members) were registered and analyzed in an additional study in 1997–1998. Continuous discussions about several points in the preliminary plan of the criteria were held during every annual meeting of the Society. A final tentative plan was discussed and decided on as the revised Japanese criteria for SS at the general meeting of the Society in Utsunomiya in September 1998.

After revision

Two years after issuing the revised criteria, an investigation to assess their availability and validity was designed and authorized using funds from a Grant-in-Aids for Scientific Research program supported by the Japan Society for the Promotion of Science. The investigation group consisted of five members: Takashi Fujibayashi, Susumu Sugai, Nobuyuki Miyasaka, Yoshio Hayashi, and Kazuo Tsubota. The assessment investigation had two parts, an availability study and a validity study. For the availability study, a questionnaire form was distributed to clinical centers that belonged to the Japanese Medical Society for Sjögren's Syndrome, after obtaining their agreement. The form consisted of seven questions covering the use of the revised criteria, the use of each examination included in the criteria, including those for minor salivary gland biopsy, lacrimal gland biopsy, sialography, a chewing gum test, the Saxon test, salivary scintigraphy, Schirmer's test, a rose bengal test, a fluorescein staining test, and anti-Ro/SS-A and anti-La/SS-B antibodies. For the validity study, 54 clinical centers responded to the request to cooperate with the assessment investigation and were enrolled. Each center was asked to register 20 consecutively applicable patients' records from their most recent files, grouped as follows: 5 patients with primary SS, 5 patients with secondary SS, 5 control patients (e.g., with simple dry mouth or dry eye but without SS) as the controls for primary SS, and 5 patients with CTD but without SS as the controls for secondary SS. It was agreed that the preliminary selection of patients with or without SS should be made following each center's clinical judgment and not using the new criteria. A total of 900 cases had been registered by March 2002. These patients included 518 cases of SS (269 cases of primary SS, 232 of secondary SS, and 17 of primary–secondary undetermined), and 382 cases of non-SS (170 primary SS controls, 180 secondary SS controls, and 32 primary–secondary undetermined SS controls. The data analysis of the records of the patients who were registered was carried out at the office of the primary investigator (T. Fujibayashi). The frequency of use of the revised criteria for diagnosing SS, and the frequency of use of each individual examination were assayed in the availability study. The sensitivity, specificity, and accuracy of the revised Japanese criteria for diagnosing SS, as well as that of each examination item in the criteria, were calculated in the validity study.

A revised version of the European criteria proposed by the American–European Consensus Group[4] was also tested for sensitivity, specificity, and accuracy by applying these criteria to the 900 Japanese cases registered in the validity study.

Results

Before revision

The values for sensitivity, specificity, and accuracy of each test item for diagnosing SS calculated from the 419 registered cases (235 SS and 184 non-SS) are listed in Tables 2–5. A minor salivary gland (MSG) biopsy was performed on 269 cases (64.2%), but a lacrimal gland (LG) biopsy was performed on only 9.1%. The cutoff values for a positive case were evaluated over the range Grade 2 to Grade 4 using Chisholm and Mason's grading.[5] Grade 3 (equivalent to 1 focus score) for the MSG biopsy showed good sensitivity (88.8%) and high specificity (97.0%) (Table 2). Sialometry with a chewing gum test[6] or with the Saxon test[7], salivary gland scintigraphy, and sialography were evaluated for the oral examination (Table 3). Sialometry alone, with either a chewing gum test or the Saxon test, did not show sufficient accuracy, while salivary gland scintigraphy showed high sensitivity (96.1%). Sialography had been used for 48.2% of the registered cases. Stage I by Rubin and Holt's stage classification[8] as a cutoff value showed a high sensitivity (86.8%) without reducing the high specificity (95.9%). The results of any single test for the ocular examination did not show adequate accuracy, and combining them resulted in little improvement in diagnosing SS (Table 4). In serological examinations, antinuclear antibody showed good purity sensitivity (88.5%) but low specificity (45.6%), while anti-La/SS-B antibody showed high specificity (96.2%) with low sensitivity (18.7%). Anti-Ro/SS-A antibody showed good sensitivity (73.4%) and specificity (77.9%) as a single test (Table 5).

The revised criteria

Each board member created draft plans for the revised diagnostic criteria after considering the results described above for each test.The sensitivity and specificity of every draft plan was calculated immediately at the board office by applying it to 419 registered cases. After intensive discussions at several board meetings and in general meetings of the Japanese Medical Society for Sjögren's Syndrome, and with some revisions and recalculations every time, the final set of revised criteria was agreed and submitted as a report to the national committee. This was published in 1999, as shown in Table 1. The calculated sensitivity, specificity, and accuracy of the revised criteria on the basis of 823 registered cases were 82.8%, 94.6%, and 87.9%, respectively (Table 6). The diagnostic ability of the old Japanese criteria[2] and the European criteria[9] were also tested using the same 823 subjects, and the results are also shown in Table 6. The revised criteria showed a little improvement over the old Japanese criteria and better results than those of the European criteria, especially in terms of sensitivity.

Table 2. Sensitivity, specificity, and accuracy of histopathological examinations of a minor salivary gland (MSG) biopsy and a lacrimal gland (LG) biopsy with various cutoff values calculated for 419 patients, including 235 SS patients and 184 non-SS control patients

Examination item	Cutoff value for positive	Sensitivity (%) TP/(TP+FN)[a]	Specificity (%) TN/(TN+FP)[a]	Accuracy (%) (TP+TN)/(TP+TN+FN+FP)[a]	No. of cases excluded
Minor salivary gland (MSG) biopsy					
(1) MSG	≧Grade 2[b]	162/169 95.9%	68/100 68.0%	230/269 85.5%	150
(2) MSG	≧Grade 3 (focus score ≧ 1)	150/169 88.8%	97/100 97.0%	247/269 91.8%	150
(3) MSG	≧Grade 4 (focus score ≧ 2)	77/169 45.6%	100/100 100%	177/269 68.8%	150
Lacrimal gland (LG) biopsy					
(4) LG	≧Grade 2	25/26 96.2%	4/12 33.3%	29/38 76.3%	381
(5) LG	≧Grade 3 (focus score ≧ 1)	18/26 69.2%	12/12 100%	30/38 78.9%	381
(6) LG	≧Grade 4 (focus score ≧ 2)	7/26 26.9%	12/12 100%	19/38 50.0%	381
(7) (2) or (5)		163/186 87.6%	137/141 97.2%	300/327 91.7%	92

[a] TP, true positive; FN, false negative; TN, true negative; FP, false positive
[b] Histopathological evaluation was expressed using Chisholm and Mason's grading (Grade 2, moderate infiltrate or less than one focus/4 mm^2; Grade 3, one focus/4 mm^2; Grade 4, more than one focus/4 mm^2 of gland tissue)

Table 3. Sensitivity, specificity, and accuracy of each test and combination of tests in oral examinations, with various cutoff values calculated, for 419 patients

Examination item	Cutoff value for positive	Sensitivity (%) TP/(TP+FN)[a]	Specificity (%) TN/(TN+FP)[a]	Accuracy (%) (TP+TN)/(TP+TN+FN+FP)[a]	No. of cases excluded
(1) Chewing gum test	≦10 ml/10 min	134/193 69.4%	76/129 58.9%	210/322 65.2%	97
(2) Saxon test	≦2 g/2 min	65/78 83.3%	18/30 60.0%	83/108 76.9%	108
(3) (1) or (2)		152/203 74.9%	75/134 56.0%	227/337 67.4%	82
(4) SG scintigraphy[b]	hypofunction	99/103 96.1%	52/90 57.8%	151/193 78.2%	226
(5) (3) and (4)		92/146 63.0%	103/123 83.7%	195/269 72.5%	150
(6) Sialography	≧Stage I[c]	112/129 86.8%	70/73 95.9%	182/202 90.1%	217
(7) Sialography	≧Stage II	84/129 65.1%	72/73 98.6%	156/202 77.2%	217
(8) Sialography	≧Stage III	17/129 13.2%	73/73 100%	90/202 44.6%	217
(9) (3) and (6)		70/131 53.4%	112/114 98.2%	182/245 74.3%	174
(10) (6) or (5)		171/198 86.4%	123/144 85.4%	294/342 86.0%	77

[a] TP, true positive; FN, false negative; TN, true negative; FP, false positive
[b] SG scintigraphy, salivary gland scintigraphy
[c] The results for sialography were expressed using Rubin and Holt's stage classification (Stage I, punctate dilatation less than 1 mm; Stage II, globular pattern with 1–2 mm; Stage III, cavitary pattern)

Table 4. Sensitivity, specificity, and accuracy of each test and combination of tests in ocular examinations, with various cutoff values, calculated for 419 patients

Examination item	Cutoff value for positive	Sensitivity (%) TP/(TP+FN)[a]	Specificity (%) TN/(TN+FP)[a]	Accuracy (%) (TP+TN)/(TP+TN+FN+FP)[a]	No. of cases excluded
(1) Schirmer's test	≦5 mm/5 min	120/206 58.3%	86/150 57.3%	206/356 57.9%	63
(2) Schirmer's test	≦10 mm/5 min	178/206 86.4%	40/150 26.7%	218/356 61.2%	63
(3) Rose bengal test	++ (score[a] ≧ 4)	83/182 45.6%	106/122 86.9%	189/304 62.2%	115
(4) Rose bengal test	+ (score[b] 1–3)	146/182 80.2%	57/122 46.7%	203/304 66.8%	115
(5) Fluorescein test	+	110/142 77.5%	51/98 52.0%	161/240 67.1%	179
(6) BUT	≦5 s	46/56 82.1%	10/34 29.4%	56/90 62.2%	329
(7) (1) and (3)		62/196 31.6%	127/134 94.8%	189/330 57.3%	89
(8) (1) and (4)		97/195 49.7%	105/133 78.9%	202/328 61.6%	91
(9) (2) and (3)		78/185 42.2%	113/127 89.0%	191/312 61.2%	107
(10) (2) and (4)		134/184 72.8%	76/126 60.3%	210/310 67.7%	109
(11) (8) or (9)		113/196 57.7%	99/134 73.9%	212/330 64.2%	89
(12) (1) and (5)		71/171 41.5%	104/128 81.3%	174/299 58.5%	120
(13) (2) and (5)		101/147 68.7%	72/112 64.3%	173/259 66.8%	160
(13) (8) or (12)		103/200 51.5%	104/136 76.5%	207/336 61.6%	83

[a]TP, true positive; FN, false negative; TN, true negative; FP, false positive
[b]Score, van Bijsterveld score for the rose bengal test

Table 5. Sensitivity, specificity, and accuracy of serological examinations calculated for 419 patients

Serological test item	Sensitivity (%) TP/(TP+FN)[a]	Specificity (%) TN/(TN+FP)[a]	Accuracy (%) (TP+TN)/(TP+TN+FN+FP)[a]	No. of cases excluded
(1) Rheumatoid factor	151/220 68.6%	114/163 69.9%	265/383 69.2%	36
(2) Antinuclear antibody	201/227 88.5%	77/169 45.6%	278/396 70.2%	23
(3) Anti-Ro/SS-A antibody	163/222 73.4%	127/163 77.9%	290/385 75.3%	34
(4) Anti-La/SS-B antibody	41/219 18.7%	150/156 96.2%	191/375 50.9%	44
(5) Hyper-gammaglobulinemia	128/183 69.9%	94/130 72.3%	223/313 71.2%	106
(6) (3) or (4)	163/222 73.4%	127/163 77.9%	290/385 75.3%	34

[a]TP, true positive; FN, false negative; TN, true negative; FP, false positive

Table 6. Diagnostic ability of various diagnostic criteria for SS applied for 823 registered patients, including 482 SS patients and 341 non-SS control patients, expressed in terms of sensitivity, specificity, and accuracy

Diagnostic criteria for SS	TP/(TP+FN)	Sensitivity (%)	TN/(TN+FP)	Specificity (%)	Accuracy (%)
(1) Revised Japanese criteria (1999)	379/458	82.8%	333/352	94.6%	87.9%
(2) Old Japanese criteria (1978)	380/462	82.3%	329/353	93.2%	87.0%
(3) European criteria (1993)	327/458	71.4%	327/351	93.2%	80.8%

After revision

The frequency of use of the revised criteria in diagnosing SS was assayed in an assessment investigation 2 years after publication. Fifty-four clinical centers were enrolled in the study, and 53 responded to the questionnaire. The revised Japanese criteria had been used at 41 centers (75.9%) for the routine diagnosis of SS. Six centers used the old Japanese criteria (11.1%), one center used the European criteria (1.9%), and two centers used other criteria (3.7%). Three centers had not used any documented criteria (5.6%). The frequency of use of each test during diagnostic examinations for SS is shown in Table 7. Serological tests for anti-Ro/SS-A and anti-La/SS-B, and Schirmer's test for the ocular examination were most commonly used. A lip biopsy (MSG biopsy) was common as a histopathological examination, and was used in 72.2% of the cases examined. A lacrimal gland biopsy was not used in 92.6% of the cases.

The sensitivity, specificity, and accuracy of the revised Japanese criteria for diagnosing SS were 96.0%, 90.5%, and 94.5%, respectively, calculated for 900 registered cases in the validity study (Fig. 1). The ability of the criteria to diagnose primary SS and secondary SS was calculated using their own controls. The sensitivity, specificity, and accuracy in diagnosing SS in patients without CTD were 96.6%, 91.8%, and 95.3%, respectively, and those for patients with CTD were 94.9%, 88.9%, and 93.3%, respectively. These results were close, and no significant difference was found between them (Fig. 1). The utility of each item in the revised criteria for diagnosing SS was also calculated, and is shown in Fig. 2. Histopathology showed the highest value

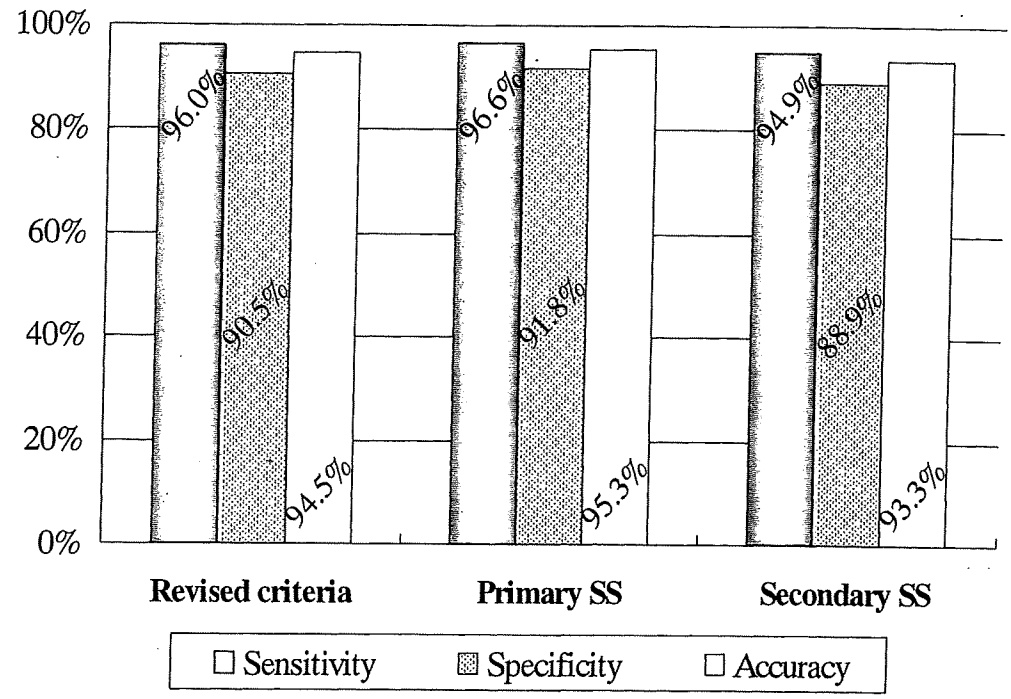

Fig. 1. The sensitivity, specificity, and accuracy of the revised Japanese criteria for diagnosing Sjögren's syndrome (SS) were calculated for 900 registered cases in the validity study. The diagnostic ability of the criteria in diagnosing primary and secondary SS was calculated each using their own control patients

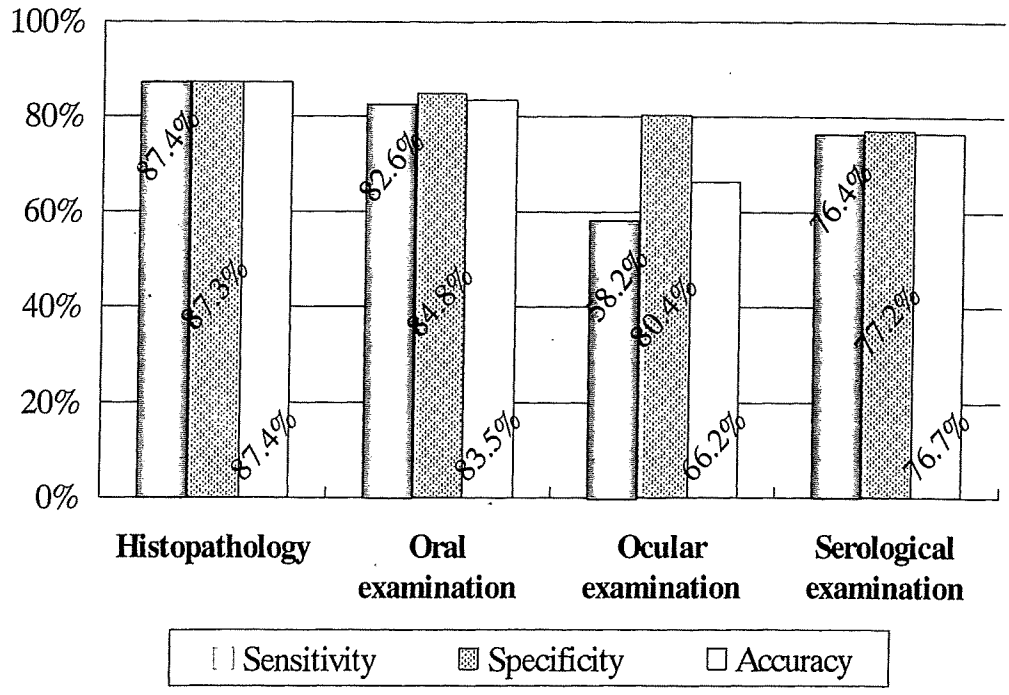

Fig. 2. The ability of each of the four examination items to diagnose SS in the revised criteria was calculated in the validity study. Histopathology showed the greatest accuracy, followed by oral examination, serological examination, and ocular examination

among the four items, followed by oral examination. The sensitivity of the ocular examination was 58.2%. Minor salivary gland (MSG) biopsy or lacrimal gland (LG) biopsy is possible in histopathology. However, the utility of histopathology depends mainly on the results of MSG biopsies because only eight examinations (0.9%) involving LG biopsy were performed without MSG biopsy. The value for the serological examination depends mainly on the results for anti-Ro/SS-A antibody because only four cases (0.4%) of anti-La/SS-B antibody were performed without anti-Ro/SS-A antibody. The sensitivity, specificity, and accuracy of each test involving an oral examination or a combination of such examinations are shown in Fig. 3. Sialography with a cutoff value in stage I showed a high sensitivity (89.1%) and specificity (91.4%). The combination of sialometry (a chewing gum test or the Saxon test) plus salivary gland scintigraphy showed good sensitivity (75.7%) and specificity (78.7%). The results for ocular examinations are

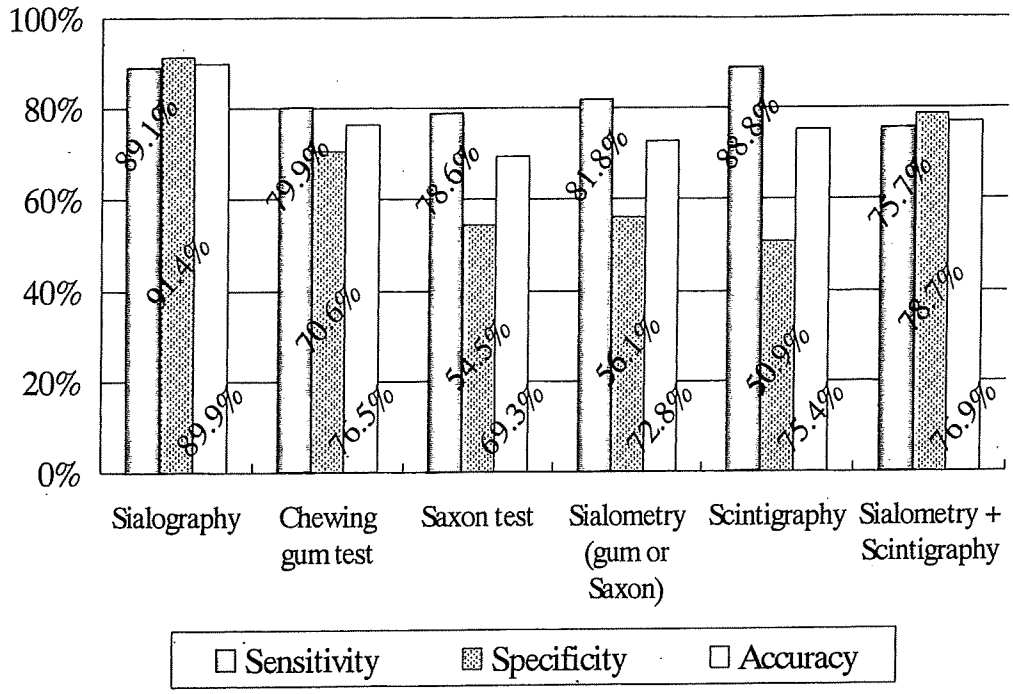

Fig. 3. The sensitivity, specificity, and accuracy of each test involving an oral examination or a combination of such examinations were evaluated in the validity study. Sialography with a cutoff value of stage I showed the greatest accuracy as a single test

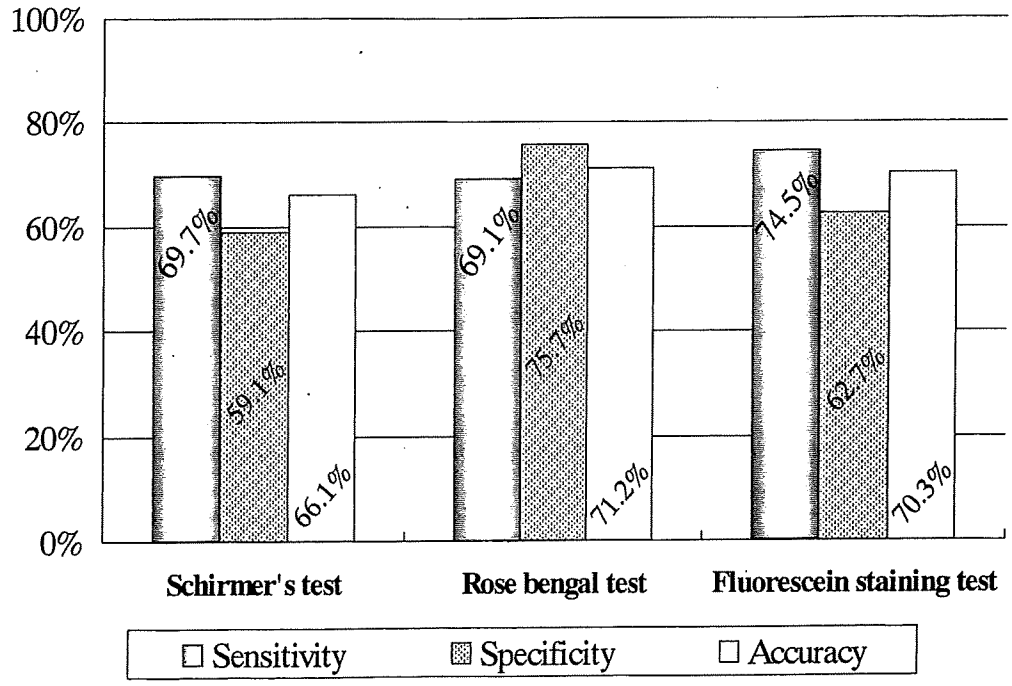

Fig. 4. The ability of three tests that involved an ocular examination to diagnose SS was calculated in the validity study. Schirmer's test with a cutoff value of 5 mm per 5 min did not show satisfactory accuracy as a single test for diagnosing SS

shown in Fig. 4. Schirmer's test with a cutoff value of 5 mm per 5 min did not show sufficient accuracy as a single test for diagnosing SS, although it was one of those used most frequently, as shown in Table 7 of the availability study. A revised version of the European criteria proposed by the American–European Consensus Group (2002) was also applied to the 900 registered cases, and the results are summarized in Table 8.

Discussion

SS is a chronic autoimmune exocrinopathy preferentially associated with dry mouth and dry eye as its major symptoms. As well as these sicca symptoms, it may be accompanied by other CTD such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis

(SSc), polymyositis/dermatomyositis (PM/DM), and mixed connective tissue disease (MCTD) in secondary SS. Some antinuclear antibodies are frequently associated with this condition. Lymphoid cell infiltration in the periductal area of the glandular tissue is a major cause of the pathological mechanism responsible for SS. However, there are no uniformly accepted world-wide criteria for the diagnosis of SS. Since there is no single infallible test to diagnose SS at present, its diagnosis is made by a combination of examinations covering the major characteristics of the disease. The revised Japanese criteria for SS consist of four items: histopathology, oral examination, ocular examination, and serological examination. It was agreed and confirmed with every participant that throughout the study, the primary classification of a patient to be registered either as having SS or as a non-SS control should be made on the basis of the investigator's clinical judgment and not on the basis of any fixed diagnostic criteria. This procedure has been also adopted in other similar investigations.[4,9–11] The revised criteria are recognized as having several differences from the old ones. (1) The revised criteria include only definite cases. (2) The subjective symptom of dry mouth or dry eyes is not indispensable. (3) The revised criteria include one item of serum autoantibody. (4) They also include salivary gland scintigraphy as a new test to evaluate salivary gland function. (5) Several combinations of tests are possible, since SS can be diagnosed when at least two of the four items are positive. As well as these points, the revised criteria contain improvements in each of the tests used for diagnosing SS. The characteristics of the revised criteria are given in Table 9, which compares them with the old Japanese criteria, and with the old European and revised versions of the European criteria (Table 9). In the old Japanese criteria, a positive cutoff value for histopathology was one focus per lobe of salivary or lacrimal gland tissue. However, this was revised to one focus per 4 mm^2, which is also used in the European criteria. The cutoff value for sialography was revised from stage II to stage I because this shift showed a large increase in sensitivity with little reduction in specificity as estimated in the study before the revision (see Table 3). This improvement was also confirmed by the validity study after the revision, which showed a high sensitivity (89.1%) and specificity (91.4%). In 2002, the cutoff value for stage I was also adopted in the revised version of the European criteria. The revised criteria can be used to diagnose primary and secondary SS. The study carried out before revision suggested the reliability of the same criteria for primary and secondary SS, and the validity study after revision confirmed this, as shown in Fig. 1. The validity study revealed that the revised criteria had a high sensitivity (96.0%) for diagnosing SS in 900 cases 2 years after their enforcement. The sensitivity had increased by 13%, but the specificity had decreased by 4% from those assayed in 823 cases before the revision . The accuracy improved by 6.6% after the revision. It is possible that this was caused by a

Table 7. Frequency of use of test items during examinations to diagnose SS by the revised Japanese criteria, summarized from a questionnaire study for 54 clinical centers

Examination tests	Frequency of use		
	Usual	Rare	Never
Minor salivary gland biopsy	72.2%	22.2%	5.6%
Lacrimal gland biopsy	3.7%	3.7%	92.6%
Sialography	37.0%	31.5%	31.5%
Chewing gum test	48.1%	20.4%	31.5%
Saxon test	35.2%	37.0%	25.9%
Salivary gland scintigraphy	40.7%	33.3%	25.9%
Schirmer's test	94.4%	1.9%	3.7%
Rose bengal test	68.5%	16.7%	14.8%
Fluorescein staining test	53.7%	25.9%	20.4%
Anti-Ro/SS-A antibody	96.3%	3.7%	0%
Anti-La/SS-B antibody	94.4%	5.6%	0%

Table 8. Sensitivity and specificity of a revised version of the European criteria proposed by the American–European consensus group applied to 900 registered cases

Items in the criteria	TP/(TP+FN)	Sensitivity (%)	TN/(TN+FP)	Specificity (%)	Accuracy (%)
(1) Revised version of the European criteria (2002)	375/441	85.0%	209/240	87.1%	85.8%
(2) For primary SS					
a. The presence of any four of the six items as long as histopathology or serology is positive	198/225	88.0%	105/111	94.6%	90.2%
b. The presence of any three of the four objective criteria items	187/219	85.4%	113/118	95.8%	89.0%
c. The classification tree procedure	nt		nt		nt
(3) For secondary SS In patients with a potentially associated disease (for instance, another well-defined connective tissue disease (CTD)), the presence of subjective symptoms plus any two from among ocular signs, histopathology, and salivary gland involvement	163/209	78.0%	112/136	82.4%	79.7%

nt, not tested

Table 9. Characteristics of the diagnostic criteria for SS in the revised Japanese version (1999), the old Japanese version (1978), the European version (1993), and the revised European version (2002)

Items, Tests in the criteria	Revised Japanese criteria (1999)[1]	Old Japanese criteria (1978)[2]	European criteria (1993)[9]	Revised version of the European criteria (2002)[4]
(1) Definite and/or probable case	Definite case	Both	Both	Definite case
(2) Subjective symptoms of dryness	Not obligatory	Essential	2 in 6 items Essential for secondary	2 in 6 items Essential for secondary
(3) Histopathology	MSG and/or LG ≧1 focus/4 mm^2	MSG and/or LG ≧1 focus/lobe	MSG ≧1 focus/4 mm^2	MSG ≧1 focus/4 mm^2
(4) Sialography	Optional in an item ≧stage I	Essential in an item ≧stage II	Optional in an item	Optional in an item ≧stage I
(5) Sialometry	Optional in an item Gum or Saxon test	In a probable case Chewing gum test	Optional in an item UWS	Optional in an item UWS
(6) Salivary gland scintigraphy	Optional in an item	Not included	Optional in an item	Optional in an item
(7) Schirmer's test	Essential in an item ≦5 mm/5 min	Optional in an item ≦10 mm/5 min	Optional in an item ≦5 mm/5 min	Optional in an item ≦5 mm/5 min
(8) Rose bengal test	Optional in an item ≧3 Bijsterveld score	Essential in an item ≧++	Optional in an item ≧4 Bijsterveld score	Optional in an item ≧4 Bijsterveld score
(9) Fluorescein staining test	Optional in an item	Optional in an item	Not included	Not included
(10) Serological examination	SS-A and/or SS-B	Not included	At least one of 3 sets	SS-A and/or SS-B
(11) Primary or secondary	Both applicable	Both applicable	Different set of items	Different set of items
(12) Exclusion criteria	Not listed	Not listed	Listed	Listed

decrease in the number of false-negative cases. This suggests that there may be a bias in the registration of patients as either SS or non-SS controls. The primary classification of the patients as SS or non-SS controls was determined using each investigator's clinical judgment. The recent changes in the criteria for the diagnosis of SS might have some effect on this judgment. However, this bias is estimated to be small, because after the revision the 900 registered cases showed similar values to those of 823 cases before revision (see Table 6) when diagnosed using the European criteria (1993). The sensitivity, specificity, and accuracy of the European criteria in these 900 cases were 77.6%, 87.6%, and 81.5%, respectively. The revised version of the European criteria (2002) showed a 7.4% improvement in sensitivity and a slight decrease in specificity over the European criteria (1993) tested using the 900 registered cases. However, the revised version of the European criteria was not the same as the revised Japanese criteria in terms of sensitivity and specificity. The availability study carried out by the questionnaire revealed a high rate of use (76%) of the revised Japanese criteria. This utilization is reasonable considering that it took place only 2 years after the enforcement of the revised criteria. Table 7 shows that the frequency of use of the test items in examinations for diagnosing SS varies. Although lacrimal gland biopsy is seldom used, some ophthalmologists may prefer it to a minor salivary gland biopsy. The flexibility in choosing test items helps to increase the availability of the criteria.

Conclusion

The Japanese criteria for diagnosing SS were revised in 1999. An assessment was carried out in 2001 to evaluate the availability and validity of the revised criteria. The availability of the revised criteria was investigated in 54 clinical centers, and the rate of use of the revised criteria was 76%. The validity of the revised criteria was evaluated using 900 enrolled cases. This evaluation showed 96.0% sensitivity, 90.5% specificity, and 94.5% accuracy in diagnosing SS.

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Activation of Epstein–Barr virus by saliva from Sjogren's syndrome patients

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SUMMARY

The aim of this study was to examine the mechanism of Epstein–Barr virus (EBV) activation by soluble factors from the inflamed salivary glands of patients with Sjogren's syndrome (SS). Saliva from SS patients was used to examine the regulation of EBV activation by an inflammatory salivary microenvironment. Transient transfection of the EBV-negative salivary gland cell line (HSY) with BZLF1, a *trans*-activating EBV gene promoter-fusion construct (Zp-luc), was used in this study. The results showed that under conditions where the BZLF1 promoter is activated by potent stimuli, SS saliva (from eight of 12 patients) exerts a significant effect on expression of the luciferase gene. A specific inhibitor of protein kinase C did not affect the SS saliva-induced Zp-luc activity, whereas treatment with inhibitors of calmodulin, calcineurin and IP_3, dose-dependently decreased this induction. Transforming growth factor β1 (TGF-β1), which is known to be expressed in SS salivary glands, dose-dependently induced Zp-luc activity. Hence, these results demonstrate the activation of EBV by SS saliva and suggest that EBV activation at the inflammatory site may occur in the presence of TGF-β1 via triggering of the mitogen-activated protein kinase (MAPK) kinase signalling pathway.

INTRODUCTION

Sjogren's syndrome (SS) is an organ-specific disorder affecting the salivary and lacrimal glands and leads to clinical symptoms of dryness of the mouth and eyes. Although the pathogenesis of SS remains unclear, there is reportedly a high incidence of Epstein–Barr virus (EBV) reactivation in SS, contributing to the initiation or perpetuation of an immune response in target organs. EBV antigens and EBV DNA have been found in infiltrating lymphocytes and salivary gland epithelial cells of SS patients.[1,2] Infectious EBV is present in both the saliva of SS patients[3–5] and culture supernatants of cell lines established from SS patients.[6] Other defined manifestations of an active EBV infection are the presence of infected B cells that can transform into B-cell lymphomas in the circulation.[7] Mariette *et al.* previously used *in situ* hybridization to detect EBV DNA in a substantial proportion of lymphoid cells and epithelial cells in salivary glands from patients with SS.[2] It has also been shown that antibodies against EBV antigens are elevated in SS sera.[8,9] Furthermore, we have recently reported an increase in the enzymatic activity of apoptotic protease, by EBV activation, to be involved in the proteolysis of 120 000 molecular weight (MW) α-fodrin autoantigen during the development of SS.[10,11] As EBV is known to induce strong immune responses,[12,13] these reports suggest that a reactivated EBV infection may play a role in SS.

EBV is a widely occurring virus of the herpes family that infects epithelial cells of the salivary glands and oropharyngeal tissue, as well as B cells. After the primary infection, the virus remains latent in the host and occasionally becomes reactivated. Reactivation of EBV requires replication of viral genes and transcriptional induction of immediate-early genes mediated by expression of the BZLF1 gene. The BZLF1 gene product, ZEBRA, is considered to first be transcribed in association

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Abbreviations: EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; HHV6, human herpesvirus 6; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PBS, phosphate-buffered saline; PKC, protein kinase C; SS, Sjogren's syndrome; TGF-β1, transforming growth factor-β1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ZEBRA, *Bam*HI-Z-DNA fragment of Epstein–Barr replication activator.

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with viral replication and to be indispensable for the reactivation of EBV.[14,15] Expression of the BZLF1 gene that encodes ZEBRA has been reported to be induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA),[16] the calcium ionophore A23187,[17] cross-linking of cell-surface immunoglobulin G (IgG),[18] n-butyrate,[19] the formation of het DNA associated with P3HR-1 superinfection,[20,21] nitric oxide (NO) inhibitor,[22] human herpesvirus 6 (HHV6) superinfection *in vitro*[23] and transforming growth factor-β1 (TGF-β1).[24] However, the physiological stimuli responsible for the EBV activation in SS have not been characterized.

Moreover, how reactivation of EBV is actually induced in lesions associated with SS, and which signalling pathways are involved in the process of viral reactivation, have not yet been clarified. To elucidate the mechanism underlying the EBV reactivation involved in the pathogenesis and progression of SS, we analysed the contribution of EBV activation by soluble factors in inflammatory saliva using a BZLF1 promoter fusion construct (Zp-luc) transfected into the human salivary gland cell line, HSY, and investigated the signalling pathways that might be involved in EBV reactivation in SS.

MATERIALS AND METHODS

Saliva samples

Saliva samples were collected from 12 patients with primary SS. All patients were seen at the Ichikawa General Hospital of Tokyo Dental College, and diagnosed according to the criteria of Fox & Saito.[25] These patients had not received glucocorticoids or immunosuppressive agents for at least 6 months prior to saliva collection.

All of the patients were women (mean age: 56 years). As age-matched controls, saliva samples were also obtained from 10 women who had no clinical evidence of systemic autoimmune disease. The samples were centrifuged at 12 000 ***g*** for 45 min and filtered through a 0·22-μm filter to remove cells, virus and particulate debris; aliquots were stored at −80°.

Cell culture, transfections and chemicals

The salivary gland epithelial cell line HSY[26] (kindly provided by Dr M. Sato of Tokushima University) was cultured at 37° in minimal essential medium (MEM) containing HEPES (10 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (FCS). The EBV-positive B-cell line, B95-8, was maintained in RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% FCS at 37° in a humidified atmosphere of 5% CO_2 in air. We used polymerase chain reaction (PCR) techniques to generate a derivative of a BZLF1 promoter (Zp) construct. The following forward and reverse oligomers were used as primers to create the promoter of the BZLF1 gene, respectively: 5′-CTGCAGCCATGCATATTTCAACTGGG-3′ and 5′-GTCGACGCAAGGTGCAATGTTTAGTG-3. The PCR-amplified promoter fragments, including the *KpnI*/*SalI* sites were cloned into the multiple cloning site of the pGL3-basic vector (Promega, Madison, WI), upstream from the luciferase gene. The Zp-luciferase gene (Zp-luc) was transfected into HSY cells using Lipofectin (Life Technologies, Grand Island, NY). The vector pGL3-basic (lacking a promoter) and the vector pGL3-control (Promega) served as negative and positive controls, respectively. Briefly, the transfection medium containing 2 μg of plasmid DNA and 20 μl of Lipofectin reagent in 200 μl of serum-free Dulbecco's modified Eagle's minimal essential medium (DMEM) was incubated for 20 min at room temperature and then diluted with serum-free DMEM to a final volume of 2 ml and added to HSY cells, plated the previous day. The transfection process occurred at 37° for 5 hr after which the DNA-containing medium was replaced with 2 ml of DMEM containing 10% FCS. TPA, calcium ionophore A23187, compound R24571 (calmodulin inhibitor), wortmaninn (IP_3-kinase inhibitor), cyclosporin A (calcineurin inhibitor), and 1-(5-isoquinolinyl sulphonyl)-2-methylpiperazine (H-7) [a protein kinase C (PKC) inhibitor] were purchased from Sigma Co. (St Louis, MO); and U0126 [a mitogen-activated protein kinase (MAPK) inhibitor] was purchased from Promega.

Luciferase assay

In order to test the Zp response to various stimuli, we used a plasmid carrying a region of Zp from −221 to +12 bp, which was cloned upstream of the coding sequence of the bacterial luciferase gene (Zp-luc): The Zp region was previously reported to contain TPA response elements. Zp-luc was transfected into the EBV-negative salivary gland cell line, HSY, which was treated with stimuli and assayed for luciferase activity. The transfected cells were incubated for 24 hr followed by stimulation with a 1 : 50 volume of saliva. TGF-β1 (Genzyme Corp., Cambridge, MA) was added to the transfected cells under the same conditions. After rinsing with phosphate-buffered saline (PBS), cells were lysed with reporter lysis buffer (Promega) and the cell lysate was analysed by the luciferase assay with the Promega kit in a Lumat (Bio-Rad, Hercules, CA). To control transfection efficiency, plasmid pRL-TK (Promega) was co-transfected with the luciferase reporter constructs at a ratio of 1 : 4. The results showed the difference in the relative efficiency of transfection between constructs to be negligible. For the assay in the presence of TPA or A23187, cells were also treated with these chemicals after transfection. The inducibility of luciferase activity was defined as the fold activity of the sample to the untreated control. Data shown are from a representative experiment carried out a minimum of three times.

Quantification of TGF-β1 in saliva by enzyme-linked immunosorbent assay

TGF-β1 concentrations in saliva were measured using the Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R & D, Minneapolis, MN). Briefly, saliva was used immediately after thawing from −80° and latent TGF-β1 was activated to immunoreactive TGF-β1. Diluted saliva samples were placed in a 96-well microtitre plate, previously coated with recombinant human TGF-β1 soluble receptor Type II (sRII). After incubation and washing, a horseradish peroxidase-conjugated secondary antibody was added. Hydrogen peroxidase and chromogen were added and colour development was measured at 450 nm using a microplate reader.

Western blot analysis

Cells were harvested, washed briefly with PBS, resuspended in buffer comprising 100 mM Tris–HCl (pH 7·6), 50 mM NaCl, 2 mM EDTA 0·5% Nonidet P-40 (NP-40), 100 μg/ml

phenylmethanesulphonyl fluoride and 1 μg/ml of each of leupeptin, pepstatin and aprotinin, and then sonicated; protein concentrations were determined using a modified Lowry assay (Bio-Rad). Equal amounts of protein in loading buffer, heated for 5 min at 100° and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel, were transferred (by electroblotting) to a nitrocellulose membrane. The membrane was stained with Ponceau S sodium salt to verify that the same amount of protein had been deposited in each lane. Anti-ZEBRA monoclonal antibody (mAb) (Dako, Carpinteria, CA) was used as a primary antibody, and horseradish peroxidase-conjugated anti-mouse IgG was used as the secondary antibody. The intensity of ZEBRA bands was measured by densitometric analysis using a colour scanner and NIH image 1.6.2. The amount of ZEBRA induced by TPA (25 ng/ml), TGF-β1 (5 ng/ml), and saliva from SS or normal individuals (1 : 50 vol/vol), with or without neutralizing anti-TGF-β1 antibody (Genzyme Corp.), was expressed as follows: [percentage of the relative amount to the negative control (TPA, TGF-β1, saliva from SS or normal individuals) ÷ (untreated)] × 100.

RESULTS

Activation of the BZLF1 promoter (Zp) in salivary gland cells

The Zp region has previously been reported to be TPA or A23187 responsive in HeLa cells and B cells.[17,27,28] In order to test the Zp activity in salivary glands, we used a plasmid carrying a Zp region (−221 to +12 bp)[28] that was cloned into an upstream portion of the luciferase gene (Zp-luc). Zp-luc was transfected into the salivary gland cell line, HSY, which had been treated with TPA or A23187, and a luciferase assay was performed. As shown in Fig. 1, luciferase activity was dose-dependently induced with TPA and A23187, even in the absence of other EBV genes. We found that this region was also responsive to TPA or A23187 in salivary gland epithelial cells.

Activation of Zp by saliva from SS patients

We next investigated whether some soluble factors are involved in the EBV reactivation that occurs in the microenvironment of SS. We collected saliva samples from 12 SS patients and 10 normal subjects, to reproduce the oral environment. As shown in Fig. 2, eight of the 12 saliva samples from SS patients showed increased luciferase activity. In contrast, luciferase activity was not increased in the normal saliva samples. These findings suggest that some humoral factors in SS saliva might play a major role in the reactivation of EBV, which in turn leads to the progression of SS.

Effects of PKC inhibitors, calcium/calmodulin-dependent protein kinase, and MAPK kinase on Zp activation by SS saliva

In B cells, the mechanism of EBV reactivation has been analysed and reported,[29,30] but the physiological signalling for EBV reactivation in SS has not been characterized. To analyse the signalling pathways occurring downstream of SS

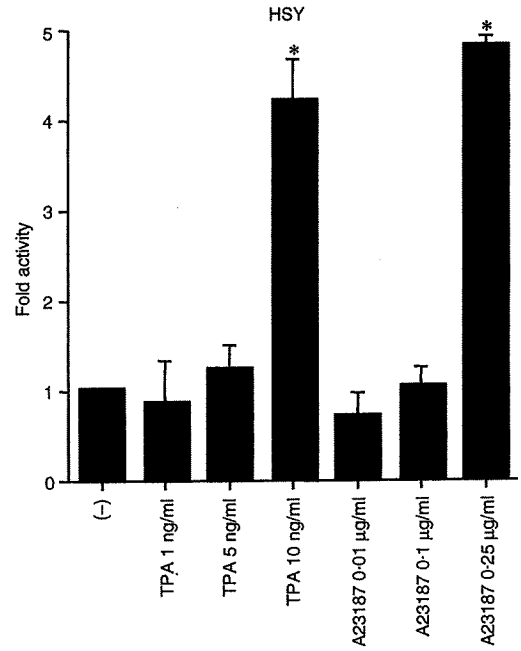

Figure 1. Activation of Zp-luc by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and A23187 in the Epstein–Barr virus (EBV)-negative salivary gland cell line, HSY. The cells were treated with either TPA (1, 5, or 10 ng/ml) or A23187 (0·01, 0·1, or 0·25 μg/ml), or given no treatment (−). After incubation for 24 hr, the cells were harvested, and luciferase activity was assayed. The bar graph illustrates fold activity. $*P < 0·05$; Mann–Whitney *U*-test. The error bars represent standard deviation.

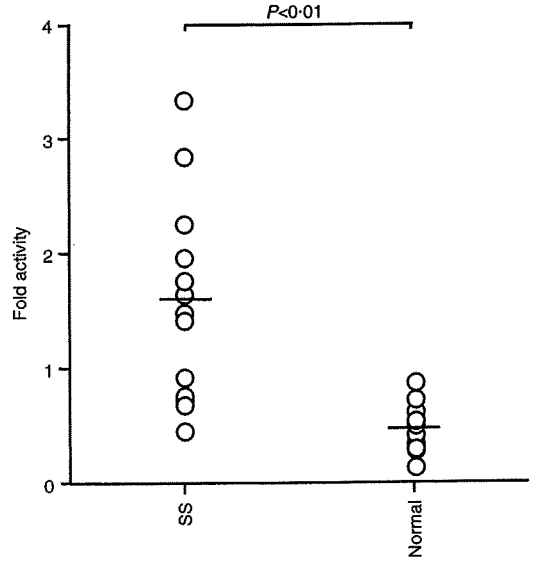

Figure 2. Measurement of luciferase activity in HSY cells transfected with Zp-luc and treated with saliva from the 12 Sjogren's syndrome patients (SS) or 10 normal controls (Normal). $P < 0·01$; Mann–Whitney *U*-test.

saliva stimulation, in our models of SS we further examined whether Zp-luc activation induced by SS saliva could be inhibited by PKC, calcium/calmodulin-dependent protein kinase or MAPK inhibitors. As shown in Fig. 3a, the PKC inhibitor did not affect luciferase activity, but the inhibitors of IP_3 (Fig. 3b), calmodulin (Fig. 3c) and calcineurin (Fig. 3d) decreased Zp-luc activity. The SS saliva used was that with the highest fold activity of Zp-luc shown in Fig. 2.

Moreover, a specific inhibitor of MAPK also reduced Zp-luc activity (Fig. 3e), suggesting that the effects of SS saliva on BZLF1 expression require calcium/calmodulin and the MAPK pathway.

Zp activation by TGF-β1

Finally, we assumed that cytokines, which are expressed in SS, might play a role in the reactivation of EBV. It is known that (in contrast to normal salivary glands) SS salivary glands express increased levels of cytokines,[31,32] but the relationship between these cytokines and EBV reactivation is not well characterized. TGF-β1, which is known to be expressed in SS, can further induce EBV reactivation in the EBV-positive B-cell lines P3HR-1, Akata[24] and B95-8.[33] To investigate cytokine efficiency in Zp activation, we conducted a luciferase assay using TGF-β1. The result indicated that 1 ng/ml of TGF-β1 increases Zp-luc activity (Fig. 4a). Next, to address the issue of the presence of TGF-β1 in saliva, we measured TGF-β1 levels

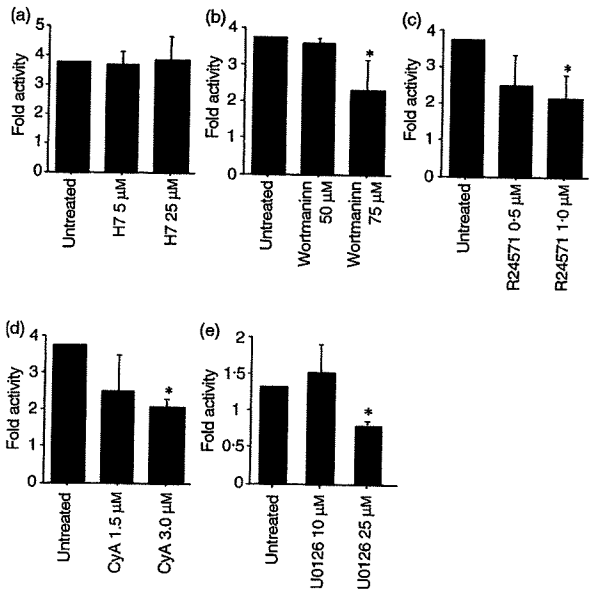

Figure 3. Effects of a protein kinase inhibitor, calmodulin antagonists, and a MAPK kinase (MEK) inhibitor on Sjogren's syndrome (SS) saliva-induced Zp-luc activation. HSY cells were pretreated for 1 hr with H7 (a), Wortmaninn (b), R24571 (c), cyclosporin A (d), or U0126 (e) before the addition of SS saliva. Cells were harvested 24 hr later, and the cell extracts were assayed for luciferase activity. This result is representative of three independent experiments. $*P < 0{\cdot}05$; Mann–Whitney *U*-test.

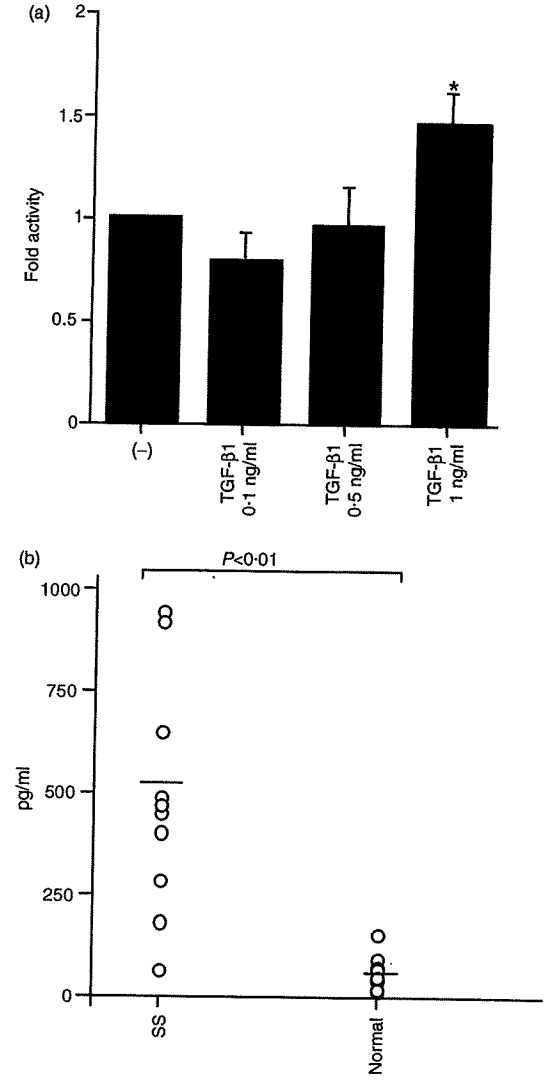

Figure 4. Effect of transforming growth factor-β1 (TGF-β1) on Zp activation. Luciferase assay of the BZLF1 promoter activities in HSY cells stimulated with TGF-β1 after transfection. Cells were harvested 24 hr later, and the cell extracts were assayed for luciferase activity. $*P < 0{\cdot}05$; Mann–Whitney *U*-test (a). TGF-β1 concentrations in saliva were measured by enzyme-linked immunosorbet assay (ELISA) (b). Saliva was used immediately after thawing from −80°. The treated samples were measured at 450 nm using a microplate reader.

using an ELISA. Figure 4(b) shows TGF-β1 concentrations to be significantly elevated in SS saliva compared with the saliva of healthy volunteers. A significant correlation was observed between the level of fold activity and the concentration of TGF-β1 in each saliva sample from SS patients ($r = 0{\cdot}75, P < 0{\cdot}01$). A concentration of 1 ng/ml TGF-β1, higher than the highest concentration in SS saliva, had 1·4-fold Zp-luc activity, whereas the SS saliva stimulated Zp-luc activity by 3·5-fold. These results demonstrate that TGF-β1 alone is not sufficient to stimulate Zp-luc, suggesting the possibility of a synergistic additional factor(s) in SS saliva. We speculate that TGF-β1