

New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjogren's syndrome

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3 **New epitopes and function of anti-M3 muscarinic acetylcholine receptor**
4 **antibodies in patients with Sjögren's syndrome**
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46 receptor, Epitopes, Function
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3 **Abstract**
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8 M3 muscarinic acetylcholine receptor (M3R) plays a crucial role in the secretion
9 of saliva from salivary glands. It is reported that some patients with Sjögren's
10 syndrome (SS) carried inhibitory auto-antibodies against M3R. The purpose of
11 this study is to clarify the epitopes and function of anti-M3R antibodies in SS. We
12 synthesized peptides encoding the extracellular domains of human-M3R
13 including the N-terminal region, the first, second, and third extracellular loops.
14 Antibodies against these regions were examined by ELISA in sera from 42 SS
15 and 42 healthy controls. For functional analysis, human salivary gland (HSG)
16 cells were pre-incubated with IgG separated from sera of anti-M3R
17 antibodies-positive SS, -negative SS, and controls for 12 hr. After loading with
18 Fluo-3, HSG cells were stimulated with cevimeline hydrochloride, and
19 intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured. Antibodies to the
20 N-terminal, first, second and third loop were detected in 42.9% (18/42), 47.6%
21 (20/42), 54.8% (23/42), and 45.2% (19/42) of SS, while in 4.8% (2/42), 7.1%
22 (3/42), 2.4% (1/42), and 2.4% (1/42) of controls, respectively. Antibodies to the
23 second loop positive SS-IgG inhibited the increase of $[Ca^{2+}]_i$ induced by
24 cevimeline hydrochloride. Antibodies to the N-terminal positive SS-IgG and
25 antibodies to the first loop positive SS-IgG enhanced it, while antibodies to the
26 third loop positive SS-IgG showed no effect on $[Ca^{2+}]_i$ as well as anti-M3R
27 antibodies negative SS-IgG. Our results indicated the presence of several B cell
28 epitopes on M3R in SS. The influence of anti-M3R antibodies on salivary
29 secretion might differ based on these epitopes.
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Introduction

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands including salivary and lacrimal glands. It is characterized by lymphocytic infiltration into exocrine glands, leading to dry mouth and eyes. A number of autoantibodies, such as anti-SS-A and SS-B antibodies, are detected in patients with SS. However, no SS-specific pathologic autoantibodies have yet been found in this condition.[1]

Data from recent studies have suggested that some patients with SS carry inhibitory autoantibodies directed against muscarinic acetylcholine receptors especially M3 muscarinic acetylcholine receptor (M3R).[1] To date, five subtypes of muscarinic acetylcholine receptors (M1R-M5R) have been identified, and M3R is expressed in exocrine glands and plays crucial roles in exocrine secretion. Acetylcholine binds to and activates M3R on salivary gland cells, causing a rise in intracellular Ca^{2+} via inositol 1, 4, 5-trisphosphate (IP3) and IP3 receptors. Consequently, the rise in intracellular Ca^{2+} activates apical membrane Cl^- channels and induces salivary secretion.[1] Activation of M3R also induces trafficking of aquaporin 5 (AQP5) to the apical membrane from the cytoplasm, which causes rapid transport of water across the cell membrane.[2] M3R has four extracellular domains; the N-terminal region, the first, second, and third extracellular loops. Among these domains, the second extracellular loop is critical for receptor activation by agonists.[3] Therefore, the second extracellular loop of M3R has been the focus of our interest, and we reported a subgroup of SS patients who had anti-M3R antibodies that recognized the second extracellular loop of M3R.[4, 5] Although these data indicate that the second

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3 extracellular loop is the target antigen, the precise epitopes are currently
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6 70 unknown. A recent study reported that the third extracellular loop represents a
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8 functional epitope bound by IgG derived from SS patients.[6]
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10 The present study was designed to clarify the precise B cell epitopes of
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12 M3R and the function of anti-M3R antibodies. For this purpose, we screened
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14 sera of SS patients for anti-M3R autoantibodies against all four extracellular
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17 75 domains of M3R by enzyme-linked immunosorbent assay (ELISA) using
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19 synthetic peptide antigens and performed functional assays of these antibodies
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21 using human salivary gland (HSG) cells. We assessed the correlation between
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23 epitopes and function and various clinical features.
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Materials and Methods

Study population

Serum samples were collected from 42 Japanese patients with SS (15 with primary SS and 27 with secondary SS) who had been followed up at the Division of Rheumatology, University of Tsukuba Hospital, Ibaraki, Japan. All patients with SS satisfied the Japanese Ministry of Health criteria for the diagnosis of SS. These criteria included four clinicopathological findings: lymphocytic infiltration of the salivary or lacrimal glands, dysfunction of salivary secretion, keratoconjunctivitis sicca, and presence of anti-SS-A or SS-B antibodies. The diagnosis of SS was based on the presence of two or more of the above items. We also recruited 42 healthy controls (HC). Approval for this study was obtained from the local ethics committee and a signed informed consent was obtained from each subject.

Synthesis of peptide antigens

We synthesized different peptides encoding the extracellular domains of human-M3R. The N-terminal of human-M3R has a 66-mer amino acid sequence, and accordingly we divided this domain into three segments. The sequences were, MTLHNNSTTSPLFPNISSSWIHSPSDAGLP for N-terminal 1, IHSPSDAGLPPGTVTHFGSYNVSRAAGNFS for N-terminal 2, and NVSRAAGNFSSPDGTTDDPLGGHTVWQV for N-terminal 3 (Sigma-Aldrich Japan, Ishikari, Japan). These three peptides were mixed and used for the peptide antigens of the N-terminal region. We also synthesized three peptides corresponding to the sequences of the three extracellular loops of human-M3R,

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3 whose sequences were FTTYIIMNRWALGNLACDLW for the first extracellular
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6 105 loop, KRTVPPGECFIQFLSEPTITFGTAI for the second, and
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8 VLVNTFCDSKIPKTFWNLY for the third (Sigma-Aldrich Japan). As a control
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10 peptide, we synthesized a peptide corresponding to the sequences of the third
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12 extracellular loop of human-M5 muscarinic acetylcholine receptor (M5R), whose
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14 sequences were STFCDKCVPTLWH (Sigma-Aldrich Japan). As a negative
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17 110 peptide, we also synthesized a 25 mer peptide whose sequence was
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20 SGSGSGSGSGSGSGSGSGSGSGSGSGSGS (Sigma-Aldrich Japan).
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22 *Enzyme-linked immunosorbent assay (ELISA)*

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24 Peptide solution (100 μ l/well at 10 μ g/ml) in 0.1 M Na₂CO₃ buffer, pH 9.6, was
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26 adsorbed onto a Nunc-Immuno plate (Nalge Nunc International, Rochester, NY)
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29 115 overnight at 4 °C, and blocked with 5% bovine serum albumin (Wako Pure
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31 Chemical Industries, Osaka, Japan) in phosphate buffered saline (PBS) for 1 hr
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33 at 37 °C. For the dose dependent curve, serum from anti-M3R antibodies positive
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35 SS and from HC were diluted at 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, and
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37 1:1600 in blocking buffer, and incubated for 2hr at 37 °C. Serum to be examined
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40 120 at 1:50 dilution in blocking buffer was also incubated for 2 hr at 37 °C. The plates
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42 were then washed six times with 0.05% Tween20 in PBS, and 100 μ l of solution
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44 of alkaline phosphatase-conjugated goat anti-human IgG (Fc; American Qualex,
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46 San Clemente, CA) diluted 1:1000 in PBS was added for 1 hr at room
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50 temperature. After nine washes, 100 μ l of p-nitrophenyl phosphate (Sigma)
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53 125 solution was added at a final concentration of 1 mg/ml as alkaline phosphatase
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55 substrate. Plates were incubated for 30 min at room temperature in the dark, and
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58 the absorbance at 405nm was measured by plate spectrophotometry.
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3 Measurements were performed in triplicate and standardized between
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5 experiments by using the absorbance value of the positive control.
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8 9 10 *Measurement of salivary secretion*

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12 We assessed salivary secretion by the Gum test. In this test, the volume of
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14 saliva is measured after chewing gum for 10 min.
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18 19 20 *Histopathological examination*

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22 Histopathological findings of the labial salivary glands were classified according
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24 to Greenspan grading.[7]
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28 29 30 *Expression of M3R mRNA in HSG cells*

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32 Total RNA was extracted from HSG cells and cDNA was synthesized by cDNA
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34 synthesis kit (Fermentas International, Ontario, Canada). PCR was performed
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36 with cDNA using the human-M3R specific primers.[2] The
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38 human-glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified
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40 to assess the cDNA yield.
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44 45 46 *Expression of M3R proteins on the cell surface of HSG cells*

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48 For immunofluorescent analysis, HSG cells were pre-cultured in 2 well chamber
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50 slides for 48 hr. Without fixation, HSG cells were incubated with the first
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52 antibodies which were anti-human M3R antibody (goat IgG, polyclonal, Santa
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54 Cruz Biotechnology, CA) or goat control IgG (Invitrogen Corporation, CA) for
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2hr. After washing, HSG cells were incubated with the second antibodies which

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3 were FITC conjugated rabbit anti-goat IgG antibodies (IgG, MP Biomedicals,
4 CA). Stained HSG cells were observed by fluorescence microscope.
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11 155 *Functional assays*

12 HSG cells (15,000 cells/well) were pre-cultured in 96-well plates for fluorescence
13 assays at 37°C for 48 hr. Then, the cells were pre-incubated with IgG fractions
14 separated from sera of anti-M3R antibodies positive 5 SS patients, anti-M3R
15 antibodies negative one SS patient, and HC by using protein G column (1.0
16 mg/ml) for 12 hr. The referral of the anti-M3R antibodies positive or negative sera
17 was on the basis of our ELISA results. IgG was washed off, and the HSG cells
18 were loaded with Fluo-3, which was a fluorescence probe for calcium, for 1 hr.
19 Fluo-3 was washed off, and then the HSG cells were analyzed. For Ca²⁺ influx
20 assay, the HSG cells were stimulated with cevimeline hydrochloride which was a
21 M3R specific agonist at a final concentration of 20 mM. Changes in intracellular
22 calcium concentrations ([Ca²⁺]_i) in HSG cells were measured by fluorescence
23 plate reader. Maximum changes of [Ca²⁺]_i (peak [Ca²⁺]_i – baseline [Ca²⁺]_i) in IgG
24 from SS patients or without IgG were shown as ratiometric data compared to
25 maximum change of [Ca²⁺]_i in HC. [2]
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48 *Statistical analysis*

49 Differences between groups were examined for statistical significance using the
50 Mann-Whitney's U-test, while differences in frequencies were analyzed by the
51 Fisher's exact probability test. A *P* value less than 0.05 denoted the presence of
52 a statistically significant difference.
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Results

Anti-M3R antibodies in patients with SS and control subjects

The average age of SS patients was 53.1 ± 13.2 y, that of HC was 33.1 ± 8.7 y ($P < 0.05$, Mann-Whitney's U-test). All of 42 SS patients were female, 22 of HC were female and 20 of HC were male. Among 27 patients with secondary SS, 11 were complicated with rheumatoid arthritis (RA), 11 with systemic lupus erythematosus (SLE), 2 with mixed connective tissue disease (MCTD), and 3 with other autoimmune diseases.

Anti-M3R antibodies were really specific for each M3R peptide, because the binding activities of sera from SS patients were dose dependent and were not in the control sera from healthy subjects. Furthermore, sera from anti-M3R antibodies positive SS did not recognize the peptide corresponding to the sequences of the third extracellular loop of human-M5R (Figure 1-A).

Antibodies to the N-terminal region were detected in 42.9% (18/42) of SS patients but only in 4.8% (2/42) of the control ($P < 0.05$, Fisher's exact probability test). Antibodies to the first extracellular loop were detected in 47.6% (20/42) of SS and 7.1% (3/42) of the control ($P < 0.05$, Fisher's exact probability test).

Antibodies to the second extracellular loop were detected in 54.8% (23/42) of SS and 2.4% (1/42) of the control ($P < 0.05$, Fisher's exact probability test).

Antibodies to the third extracellular loop were detected in 45.2% (19/42) of SS and 2.4% (1/42) of the control ($P < 0.05$, Fisher's exact probability test). The

frequencies and titers of anti-M3R antibodies against all extracellular domains were significantly higher in SS patients than the control ($P < 0.05$, Fisher's exact probability test for frequencies, Mann-Whitney's U-test for titers) (Figure 1-B).

B cell epitopes on the M3R

Table 1 lists the epitopes of anti-M3R antibodies in patients with SS. Of the 42 SS patients, 28 had anti-M3R antibodies reactive to at least one B cell epitope on the M3R, while the other 14 SS patients did not have any anti-M3R antibodies. Antibodies to one B cell epitope on the M3R (N-terminal, first extracellular loop, second extracellular loop, and third extracellular loop) were detected in one, two, two, and one SS patients out of 28 patients, respectively. Antibodies reactive to two B cell epitopes (N-terminal and first extracellular loop, N-terminal and second extracellular loop, first and second extracellular loop, second and third extracellular loop) were detected in one, one, two, and two SS patients, respectively. Two SS patients showed the presence of antibodies to three B cell epitopes (N-terminal and second and third extracellular loop, first and second and third extracellular loop). In 50% of the SS patient (14/28), antibodies reactive to all four B cell epitopes were detected. Based on these results, we concluded that anti-M3R antibodies had several B cell epitopes on the extracellular domains of M3R, and that some SS patients carried anti-M3R antibodies that recognized several extracellular domains of M3R.

Correlation between anti-M3R antibodies and various clinicopathological features

Disease duration of SS was shorter among anti-M3R antibodies-positive SS (7.3 ± 7.6 y) than -negative SS (15.5 ± 11.1 y, $p < 0.05$, Mann-Whitney's U-test).

The positivity for anti-SS-A antibody and the IgG value in serum was more prevalent and higher among anti-M3R antibodies-positive SS than -negative SS

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3 (p<0.05, Fisher's exact probability test and Mann-Whitney's U-test). In contrast,
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5 there were no differences in age, positivity for anti-SS-B antibody and
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7 rheumatoid factor, tear volume by Schirmer test, saliva volume by Gum test,
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9 extra-glandular involvement, and Greenspan grading, between anti-M3R
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11 antibodies-positive and -negative SS (Table 2). There is no significant
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13 230 relationship between each B cell epitope and clinical characteristics such as
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15 saliva secretion.
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22 *Expression of M3R mRNA and proteins in HSG cells*

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25 235 PCR products revealed the expression of M3R mRNA in HSG cells used in the
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27 present study. The expected PCR product for M3R was detected at 201 bp
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29 (Figure 2-A). Moreover, M3R proteins were detected on HSG cells stained with
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31 anti-human M3R antibody, whereas they were not found with control IgG (Figure
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33 2-B). These results indicated that HSG cells expressed M3R molecules on their
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35 surface.
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41 *Functional roles of anti-M3R antibodies*

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43 IgG derived from two SS patients positive for anti-M3R antibodies to the second
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45 extracellular loop inhibited the increase in $[Ca^{2+}]_i$ induced by cevimeline
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47 hydrochloride 16% and 25%, respectively (P<0.05, vs IgG derived from HC,
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49 245 Mann-Whitney's U-test) (Figures 3-C, 3-D and 4). In contrast, IgG derived from
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51 SS patients positive for antibodies to the N-terminal and the first extracellular
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53 loop enhanced the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride 14%
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55 and 15%, respectively (P<0.05, vs IgG derived from HC, Mann-Whitney's U-test)
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60 250 (Figures 3-A, 3-B, and 4). IgG derived from a SS patient positive for antibodies to

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the third extracellular loop had no effect on $[Ca^{2+}]_i$ as well as IgG derived from anti-M3R antibodies-negative SS patient (Figures 3-E and 4).

For Peer Review

Discussion

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6 Recently, anti-M3R antibodies have been the focus of interest in rheumatology,
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8 because of their potential pathogenic role, use as diagnostic markers, and being
9 255 therapeutic targets in patients with SS.[1] Several methods have been used to
10 detect anti-M3R antibodies in SS patients.[1] In functional assays using smooth
11 muscles, IgG fractions from patients with SS (SS-IgG) inhibited
12 carbachol-evoked or nerve-evoked bladder or colon contractions.[8, 9] In
13 salivary gland cells, SS-IgG inhibited the rise in $[Ca^{2+}]_i$ induced by carbachol,
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15 and also inhibited pilocarpine-induced AQP5 trafficking to the apical membrane
16 from the cytoplasm.[2] The inhibitory actions of SS-IgG on the rise in $[Ca^{2+}]_i$ was
17 acutely reversible.[10] Anti-M3R antibodies from SS patients can be detected by
18 immunofluorescent analysis using rat lacrimal glands [11], and by flow cytometry
19 using the M3R-transfected CHO cell line.[12] Moreover, anti-M3R antibodies in
20 260 sera of SS patients were detected by ELISA using synthetic peptides or
21 recombinant proteins of the second extracellular loop of M3R.[13] We reported
22 previously the presence of anti-M3R antibodies in a group of patients with SS,
23 which recognized the second extracellular loop by ELISA using synthetic
24 peptides.[4, 5]
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46 In the present study, we established a standard method to detect
47 anti-M3R antibodies that can be used for screening large patient populations.
48 Functional assays and flow cytometry are too laborious for routine use. Although
49 ELISA is easy, the results from some ELISA systems used for screening
50 anti-M3R antibodies differ widely with regard to the prevalence of anti-M3R
51 275 antibodies (from 11 to 90%).[4, 14] Furthermore, Cavill *et al.* [15] reported failure
52 to detect anti-M3R antibodies by ELISA using synthetic peptides. In the present
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3 study, we reported higher frequencies and titers of anti-M3R antibodies against
4 all extracellular domains in SS patients than the control. The prevalence of
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6 anti-M3R antibodies against the second extracellular loop in SS (55%)
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280 anti-M3R antibodies against the second extracellular loop in SS (55%)
determined in the present study was much higher than that reported in our
previous study (11%).[4] The reason for this difference is probably related to the
change in the methodology, such as increased sensitivity resulting from purity of
the synthetic peptides, modification of the washing procedure, or other factors
introduced in the modified ELISA system.

In the present study, we also determined the precise B cell epitopes of
M3R molecules. B cell epitopes in the present study are areas including peptides
recognized by anti-M3R antibodies, although we do not know that these linear
peptides are really conformational epitopes or not. However, we showed
anti-M3R antibodies against these linear epitopes exactly influenced Ca influx
via M3R in HSG cells. Therefore, we suggest that these linear peptides might
consist of the conformational epitopes on the M3R. Several B cell epitopes were
identified on the extracellular domains, and some SS patients were reactive to
several extracellular domains other than the second extracellular loop. The
second extracellular loop of M3R has been the focus of our interest in epitopes
and function of anti-M3R antibodies.[4, 5, 9, 10] Recently, Koo *et al.* [6] reported
that the third extracellular loop represents a functional epitope bound by SS-IgG.
In contrast to these results, we found in the present study that antibodies to the
second extracellular loop of M3R inhibited the increase of $[Ca^{2+}]_i$ induced by
cevimeline hydrochloride in a functional assay using HSG cells. This inhibitory
effect of anti-M3R antibodies might explain the reduction in salivary secretion in
some SS patients. Our data also demonstrated that antibodies against the third

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3 extracellular loop did not have an effect on the increase in $[Ca^{2+}]_i$, while
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5 antibodies against the N-terminal and first extracellular loop enhanced the
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9 305 increase in $[Ca^{2+}]_i$. These results indicate that the effects of anti-M3R antibodies
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11 on the secretion of saliva could be different from these epitopes, although further
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13 experiments using antibodies from more patients are necessary.
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16 Although the molecular mechanism on the difference among individual B
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18 cell epitopes have not elucidated, we could propose the following three
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20 310 possibilities. The first possibility is that antibodies against the second
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22 extracellular domain of M3R directly inhibit the intracellular signal pathway,
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24 resulting in the decrease of Ca^{2+} influx and reduction of saliva. In contrast,
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26 antibodies against N-terminal region and the first extracellular domain of M3R
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28 might enhance the intracellular signaling and increase of Ca^{2+} influx. The second
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31 315 is that anti-M3R antibodies binding to the second extracellular domain could
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33 inhibit M3R agonist, then antibodies indirectly suppress the stimulation of Ca^{2+}
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35 influx. The third is that anti-M3R antibodies influence on the expression of M3R
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37 molecules on HSG. Some antibodies which targets are N-terminal region or the
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39 first extracellular loop of M3R may be able to up-regulate the expression of M3R
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43 320 and enhance Ca^{2+} influx, whereas the other antibodies against the second
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45 extracellular domain might down-regulate the expression of M3R on HSG,
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47 resulting in reduction of Ca^{2+} influx. Exactly, it has been reported that the
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49 expression of M3R in salivary glands could be affected by anti-M3R antibodies in
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51 patients with SS.[1] Further experiments on the effect of anti-M3R antibodies on
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55 325 M3R signaling, the binding to M3R agonist, and the influence on M3R
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57 expression should shed light on the mechanism of the different function of
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59 anti-M3R antibodies.
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We reported previously the presence of anti-M3R antibodies that recognized the second extracellular loop in SS patients but not in patients with RA or SLE, suggesting that anti-M3R antibodies could be potentially used as diagnostic markers for SS.[4] However, Kovacs *et al.* [14] reported the detection of anti-M3R antibodies in 35% of their RA patients and 32% of SLE. These conflicting results emphasize the need to examine the precise prevalence of anti-M3R antibodies in other autoimmune diseases using our modified ELISA system.

The correlation between anti-M3R antibodies and clinical features is still unclear. The previous study reported leukopenia was more common in anti-M3R antibodies-positive than in -negative patients with primary SS.[14] Our observations in the present study showed that the positivity for anti-SS-A antibody and IgG values in serum was more prevalent and higher in anti-M3R antibodies-positive SS patients than -negative SS patients. The disease duration of SS was shorter among anti-M3R antibodies-positive SS than -negative SS, however there was no difference in other clinical and histological features between anti-M3R antibodies-positive and -negative SS patients. We could not detect any significant relationship between each B cell epitope and clinical characteristics such as saliva secretion.

In conclusion, these findings support the notion of presence of several B cell epitopes on M3R in SS patients and that some SS patients are reactive to several extracellular domains of the M3R. It is possible that some anti-M3R antibodies alter salivary secretion in SS via M3R, and especially antibodies against the second extracellular loop of the M3R could suppress the increase in $[Ca^{2+}]_i$ induced by agonists of the M3R, resulting in reduction of salivary

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3 secretion. Therefore, anti-M3R antibodies might play pathogenic roles in salivary
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6 secretion abnormalities characteristic of patients with SS.
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Figure Legends

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Figure 1.

(A) The dose dependent curve on anti-M3R antibodies.

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M3R and M5R peptide-specific absorbance values at 405 nm (Δ absorbance) were calculated for each serum sample by subtracting the absorbance value of the negative peptide from that of the peptides encoding the extracellular domains of human-M3R and M5R. The clear dose response of M3R peptide-specific absorbance to changes on serum concentrations was shown in serum from anti-M3R antibodies positive Sjögren's syndrome (SS), but not in serum from healthy control (C). The clear dose response of the third extracellular loop of M5R-specific absorbance to changes on serum concentrations was not shown in serum from anti-M3R antibodies positive SS.

(B) Anti-M3R antibodies in patients with SS and control subjects.

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M3R peptide-specific absorbance values at 405 nm (Δ absorbance) in Sjögren's syndrome (SS) and healthy control (C). The cut-off level between negative and positive values was the mean+2 SD value of the normal control (gray line). The prevalence and titers of anti-M3R antibodies against all extracellular domains were significantly higher in patients with SS than control subjects (Fisher's exact probability test for prevalence, Mann-Whitney's U-test for titers). N; N-terminal region, 1st; first extracellular loop, 2nd; second extracellular loop, 3rd; third extracellular loop.

Figure 2. (A) Expression of M3R mRNA in HSG cells.

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(B) Expression of M3R proteins on the surface of HSG cells detected by immunofluorescent analysis.

430 **Figure 3.**

Functional analysis of anti-M3R antibodies in SS patients.

(A) and (B)

IgG derived from SS patient with anti-M3R antibodies to the N-terminal region and the first extracellular loop enhanced the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride 14% and 15 %, respectively, compared to IgG from healthy control (HC). The traces were representative traces, which were performed in triplicate and three independent experiments with each IgG. HSG cells were stimulated with cevimeline hydrochloride (20 mM) at time 10 seconds. IgG⁻; without IgG, HC; IgG derived from healthy control, M3R⁻SS; IgG derived from SS patient negative for anti-M3R antibodies, N⁺SS; IgG derived from SS patient positive for anti-M3R antibodies to the N-terminal region, 1^{st+}SS; IgG derived from SS patient positive for anti-M3R antibodies to the first extracellular loop.

(C) and (D)

IgG derived from two SS patients positive for antibodies to the second extracellular loop inhibited the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride 16% and 25%, respectively, compared to IgG from HC. The traces were representative traces, which were performed in triplicate and three independent experiments with each IgG. 2^{nd+}SS; IgG derived from SS patient positive for anti-M3R antibodies to the second extracellular loop.

(E)

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3 IgG derived from SS patient positive for antibodies to the third extracellular loop
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5 had no effect on the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride. The
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7 traces were representative traces, which were performed in triplicate and three
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11 455 independent experiments with each IgG. 3^{rd+}SS; IgG derived from SS patient
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13 positive for antibodies to the third extracellular loop.
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18 **Figure 4.**

19 **Summary of B cell epitopes on M3R and the function of anti-M3R** 20 21 22 460 **antibodies in SS patients.** 23

24 Mean \pm SD values of maximum change in $[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$ – baseline $[Ca^{2+}]_i$)
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26 induced by cevimeline hydrochloride in IgG from SS patients or without IgG were
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28 shown as a ratio compared to maximum change of $[Ca^{2+}]_i$ in healthy control (HC).
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30 The maximum change in HC was described as 1.0. Data were averaged of
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34 465 triplicate and three independent experiments. *; $P < 0.05$, vs IgG derived from HC,
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36 Mann-Whitney's U-test, HC, IgG⁻, M3R⁻SS, N⁺SS, 1^{st+}SS, 2^{nd+}SS, and 3^{rd+}SS;
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38 the same as in Figure 3.
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Table 1
B cell epitopes on the M3R

B cell epitopes on the M3R					
Number of B cell epitopes on the M3R	N	1st	2nd	3rd	Number of cases
	+	-	-	-	1 case
1	-	+	-	-	2 cases
	-	-	+	-	2 cases
	-	-	-	+	1 case
2	+	+	-	-	1 case
	+	-	+	-	1 case
	-	+	+	-	2 cases
	-	-	+	+	2 cases
3	+	-	+	+	1 case
	-	+	+	+	1 case
4	+	+	+	+	14 cases
Total number of cases					28 cases

N; N-terminal, 1st; first extracellular loop, 2nd; second extracellular loop, 3rd; third extracellular loop, +; positive for anti-M3R antibodies, -; negative for anti-M3R antibodies

Table 2
Clinicopathological Features in Anti-M3R Antibodies-
Positive SS and -Negative SS Patients

	Positive SS N=28	Negative SS N=14	P Value
Primary/Secondary	12/16	3/11	N.S
Age (y)	51.4 ± 12.1	56.4 ± 12.1	N.S
Disease duration (y)	7.3 ± 7.6	15.5 ± 11.1	P<0.05
Anti-SSA (%)	92.9	57.1	P<0.05
Anti-SSB (%)	21.4	14.3	N.S
Rheumatoid factor (%)	46.4	50.0	N.S
IgG (mg/dl)	2013 ± 767	1427 ± 515	P<0.05
Extra-glandular involvements in primary SS (%)	83.3	66.7	N.S
Schirmer test (mm/5 min)	4.4 ± 6.2	4.2 ± 5.0	N.S
Gum test (ml/10 min)	8.3 ± 7.8	8.8 ± 5.1	N.S
Histological examination (Greenspan grading)	3.1 ± 0.5	3.0 ± 0.8	N.S

N.S; not statistically significant

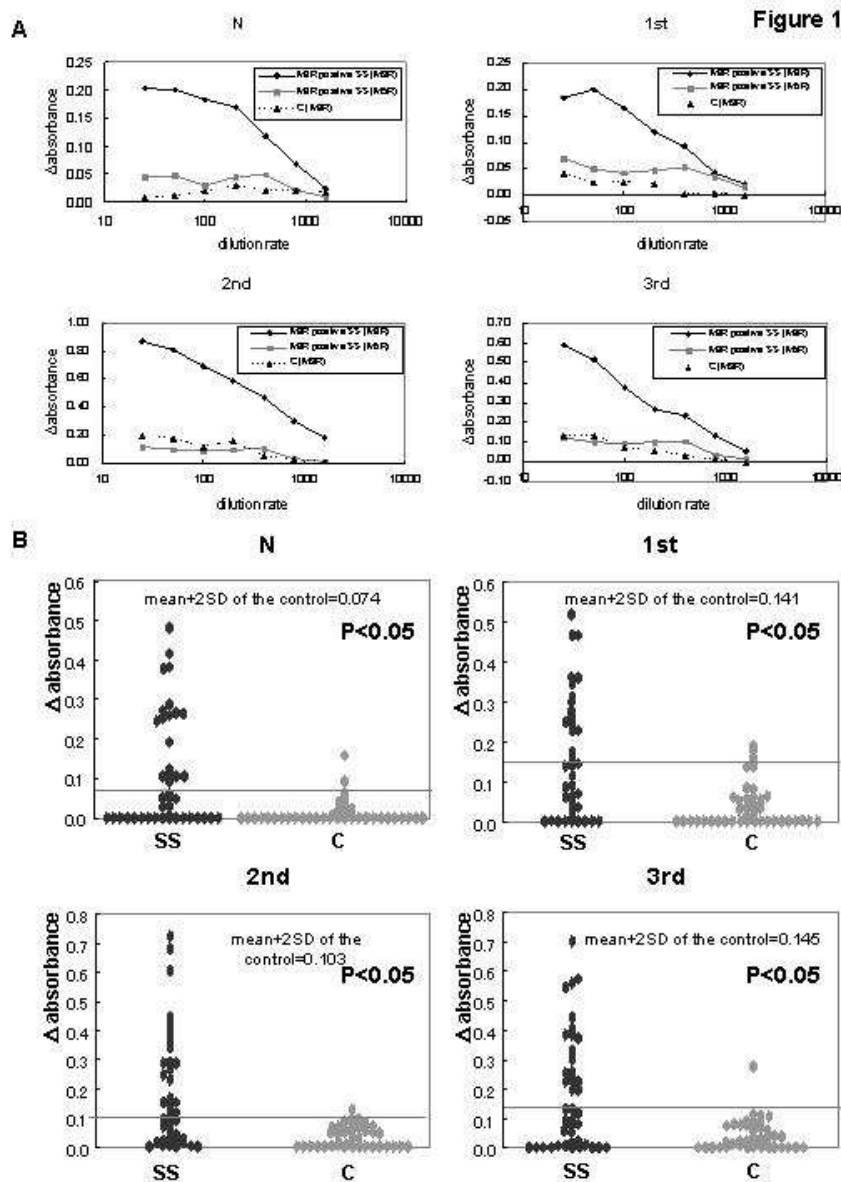


Figure 1.

(A) The dose dependent curve on anti-M3R antibodies.

M3R and M5R peptide-specific absorbance values at 405 nm (Δ absorbance) were calculated for each serum sample by subtracting the absorbance value of the negative peptide from that of the peptides encoding the extracellular domains of human-M3R and M5R. The clear dose response of M3R peptide-specific absorbance to changes on serum concentrations was shown in serum from anti-M3R antibodies positive Sjögren's syndrome (SS), but not in serum from healthy control (C). The clear dose response of the third extracellular loop of M5R-specific absorbance to changes on serum concentrations was not shown in serum from anti-M3R antibodies positive SS.

(B) Anti-M3R antibodies in patients with SS and control subjects.

M3R peptide-specific absorbance values at 405 nm (Δ absorbance) in Sjögren's syndrome (SS) and healthy control (C). The cut-off level between negative and positive values was the mean+2 SD value of the normal control (gray line). The prevalence and titers of anti-M3R antibodies against all

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extracellular domains were significantly higher in patients with SS than control subjects (Fisher's exact probability test for prevalence, Mann-Whitney's U-test for titers). N; N-terminal region, 1st; first extracellular loop, 2nd; second extracellular loop, 3rd; third extracellular loop.

190x254mm (72 x 72 DPI)

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Figure 2

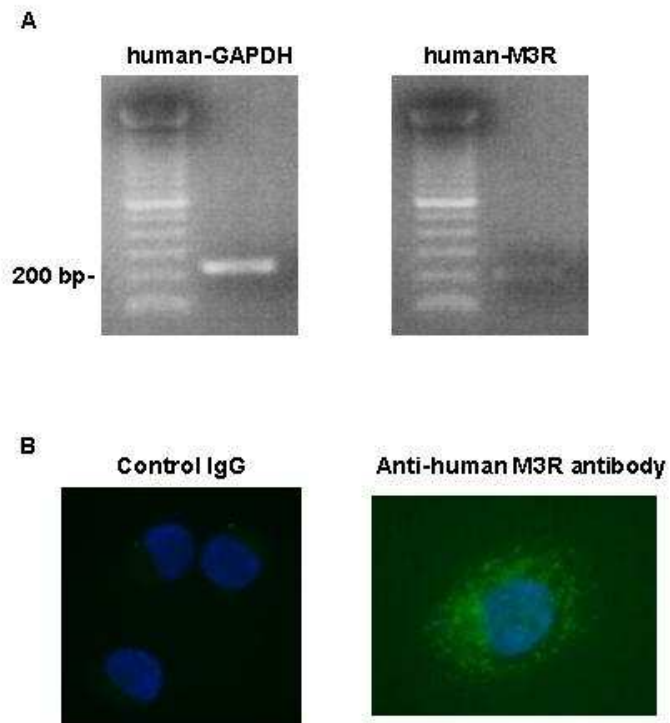


Figure 2.

(A) Expression of M3R mRNA in HSG cells.

(B) Expression of M3R proteins on the surface of HSG cells detected by immunofluorescent analysis.

190x254mm (72 x 72 DPI)

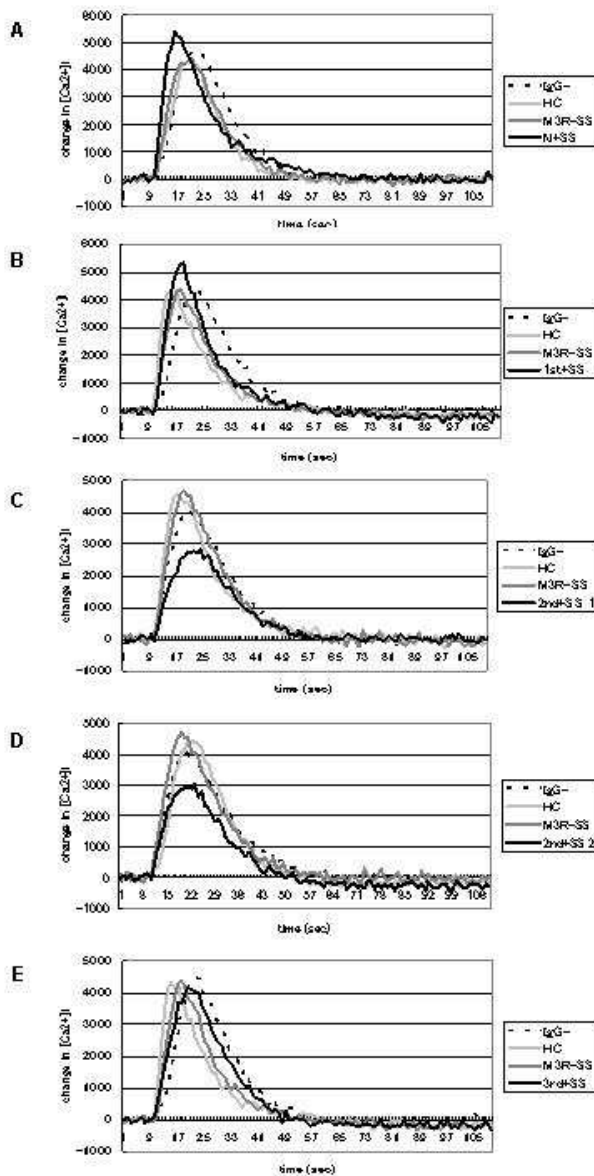


Figure 3

Figure 3.
Functional analysis of anti-M3R antibodies in SS patients.
(A) and (B)

IgG derived from SS patient with anti-M3R antibodies to the N-terminal region and the first extracellular loop enhanced the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride 14% and 15 %, respectively, compared to IgG from healthy control (HC). The traces were representative traces, which were performed in triplicate and three independent experiments with each IgG. HSG cells were stimulated with cevimeline hydrochloride (20 mM) at time 10 seconds. IgG-; without IgG, HC; IgG derived from healthy control, M3R-SS; IgG derived from SS patient negative for anti-M3R antibodies, N+SS; IgG derived from SS patient positive for anti-M3R antibodies to the N-terminal region, 1st+SS; IgG derived from SS patient positive for anti-M3R antibodies to the first extracellular loop.

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3 (C) and (D)

4 IgG derived from two SS patients positive for antibodies to the second extracellular loop inhibited
5 the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride 16% and 25%, respectively, compared
6 to IgG from HC. The traces were representative traces, which were performed in triplicate and three
7 independent experiments with each IgG. 2nd+SS; IgG derived from SS patient positive for anti-M3R
8 antibodies to the second extracellular loop.

9 (E)

10 IgG derived from SS patient positive for antibodies to the third extracellular loop had no effect on
11 the increase in $[Ca^{2+}]_i$ induced by cevimeline hydrochloride. The traces were representative traces,
12 which were performed in triplicate and three independent experiments with each IgG. 3rd+SS; IgG
13 derived from SS patient positive for antibodies to the third extracellular loop.

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Figure 4

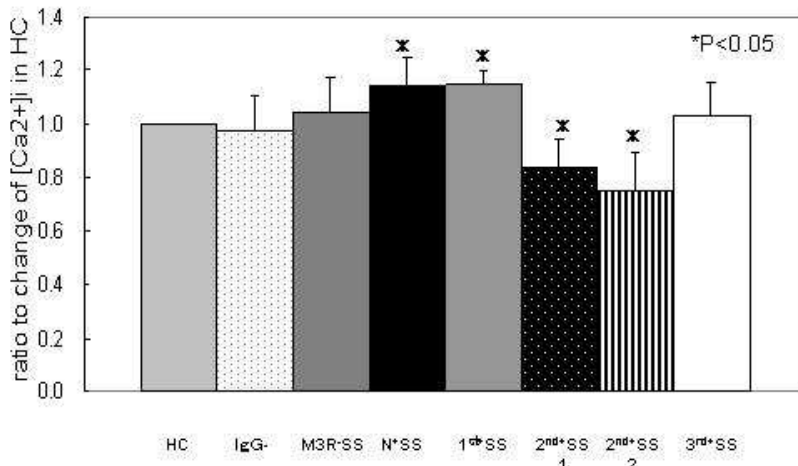


Figure 4.

Summary of B cell epitopes on M3R and the function of anti-M3R antibodies in SS patients. Mean \pm SD values of maximum change in [Ca²⁺]_i (peak [Ca²⁺]_i – baseline [Ca²⁺]_i) induced by cevimeline hydrochloride in IgG from SS patients or without IgG were shown as a ratio compared to maximum change of [Ca²⁺]_i in healthy control (HC). The maximum change in HC was described as 1.0. Data were averaged of triplicate and three independent experiments. *; P<0.05, vs IgG derived from HC, Mann-Whitney's U-test, HC, IgG-, M3R-SS, N+SS, 1st+SS, 2nd+SS, and 3rd+SS; the same as in Figure 3.

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