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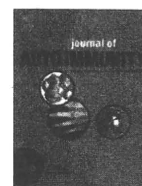

Pathogenic role of immune response to M3 muscarinic acetylcholine receptor in Sjögren's syndrome-like sialoadenitis

Mana Iizuka [a,1], Ei Wakamatsu [a,b,1], Hiroto Tsuboi [a], Yumi Nakamura [a,c], Taichi Hayashi [a], Minoru Matsui [d], Daisuke Goto [a], Satoshi Ito [a], Isao Matsumoto [a], Takayuki Sumida [a,*]

[a] Division of Clinical Immunology, Doctoral Programs in Clinical Sciences, Graduate School Comprehensive Human Science, University of Tsukuba, 1-1-1 Tenodai, Tsukuba City, Ibaraki 305-8575, Japan
[b] Department of Pathology, Harvard Medical School, Boston, MA 02115, USA
[c] Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan
[d] Department of Clinical Research and General Medicine, Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan

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ABSTRACT

The aim of this study was to clarify the role of the immune response to muscarinic type 3 receptor (M3R) in the pathogenesis of Sjögren's syndrome (SS). $M3R^{-/-}$ mice were immunized with murine M3R peptides and their splenocytes were inoculated into $Rag1^{-/-}$ ($M3R^{-/-} \rightarrow Rag1^{-/-}$) mice. $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high serum levels of anti-M3R antibodies and low saliva volume. Histological examination showed marked infiltration of mononuclear cells in the salivary glands and immunohistochemistry demonstrated that the majority of these cells were $CD4^+$ T cells with a few B cells and several IFN-γ- and IL-17-producing cells. Apoptotic cells were present in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, transfer of only $CD3^+$ T cells from $M3R^{-/-}$ immunized with M3R peptides into $Rag1^{-/-}$ mice resulted in cell infiltration and destruction of epithelial cells in the salivary glands, suggesting that M3R reactive $CD3^+$ T cells play a pathogenic role in the development of autoimmune sialoadenitis. Our findings support the notion that the immune response to M3R plays a crucial role in the pathogenesis of SS-like autoimmune sialoadenitis.
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1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by infiltration of lymphocytes into lacrimal and salivary glands, and clinically by dry eyes and dry mouth [1]. Immunohistochemical studies have shown that most infiltrating lymphocytes around the labial salivary and lachrymal glands are CD4-positive αβ T cells [2]. B cells are also detected in inflammatory lesions and act as antigen-presenting cells (APC) and produce autoantibodies such as anti-Ro/SSA and anti-La/SSB antibodies. Previous studies analyzed autoantigens recognized by T cells infiltrating the labial salivary glands of patients with SS and several candidate autoantigens such as Ro/SSA 52 kDa, [3] α-amylase, [4] heat shock protein, T cell receptor BV6 [5] and M3 muscarinic acetylcholine receptor (M3R) [6,7] have been identified.

M3R is the major muscarinic acetylcholine receptor in the salivary glands. Studies in M3R knockout ($M3R^{-/-}$) mice showed the involvement of these receptors in saliva secretion [8], suggesting that dysfunction of the M3R signaling in salivary glands can lead to a decrease in salivary secretion. The presence and specificity of anti-M3R antibodies in SS have been investigated using enzyme-linked immunosorbent assay (ELISA) or flow cytometry assay [9,10]. Robinson et al. [11] demonstrated that human anti-M3R antibodies reduced salivary secretion in NOD $Ig\mu^{null}$ mice. We also reported previously the presence of M3R reactive T cells in some patients with SS [12]. At this stage, however, it is not clear whether the immune response to M3R plays a role in the onset and progression of SS.

Amagai et al. [13] described a mouse model of pemphigus vulgaris (PV) using the combination of desmoglein 3 knockout ($Dsg3^{-/-}$) mice without Dsg3 and adoptive splenocytes transfer to lymphocyte-free Dsg3-positive $Rag2^{-/-}$ mice ($Dsg3^{-/-} \rightarrow Rag2^{-/-}$). In the $Dsg3^{-/-} \rightarrow Rag2^{-/-}$ mice, the Dsg3 molecule induces a powerful immune response and the cells break self-tolerance and attack Dsg3 autoantigens. The same approach might be applicable to T cell-mediated autoimmune disease as well as antibody-mediated disease such as PV.

Our research interest is the pathogenic role of the immune response against M3R in patients with SS. In the present study, we

* Corresponding author. Tel.: +81 29 853 3221; fax: +81 29 853 3222.
E-mail address: tsumida@md.tsukuba.ac.jp (T. Sumida).
[1] These authors contributed equally to this work.

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immunized $M3R^{-/-}$ mice with murine M3R peptides and transferred their splenocytes into $Rag1^{-/-}$ mice ($M3R^{-/-} \rightarrow Rag1^{-/-}$). We succeeded in establishing M3R-induced SS-like sialoadenitis in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice as a new murine model of SS. The results also showed that $CD3^{+}$ T cells play a crucial role in the generation of autoimmune sialoadenitis. We also discuss the possible molecular mechanism of M3R-induced autoimmunity in SS.

2. Materials and methods

2.1. Mice

C57BL/6j (B6) mice ($M3R^{+/+}$) were purchased from Charles River Laboratory (Yokohama, Japan). $Rag1^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $M3R^{-/-}$ mice, which were generated from B6 mice, were kindly supplied by Dr. Matsui (Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Resource Center. All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at Tsukuba University.

2.2. Synthesized peptides encoding M3R extracellular regions

Six types of peptides encoding murine M3R extracellular domains: N-terminus 1 (MTLHSNSTTSPLFPNISSSWVHSPSEAGLP, N1), N-teminus 2 (VHSPSEAGLPLGTVSQLDSYNISGTSGNFS, N2), N-terminus 3 (NISQTSGNFSSNDTSSDPLGGHTIWQV, N3), 1st extracellular loop (FTTYIIMNRWALGNLACDLW, 1st), 2nd extracellular loop (QYFVGK RTVPPGECFIQFLSEP, 2nd) and 3rd extracellular loop (VLVNTF CDSCIPKTYWNLGY, 3rd), were synthesized chemically by a solid-phase procedure and purified by high performance liquid chromatography (AnyGen, Korea).

2.3. Immunization of mice

$M3R^{+/+}$ and $M3R^{-/-}$ mice were immunized intradermally at the base of the tail with a mixture of free-form extracellular peptides (each 20 μg) in Incomplete Freund's Adjuvant (IFA, Difco, Detroit, MI) containing 250 μg of inactivated Mycobacterium tuberculosis (H37RA, Difco). Pertussis toxin (500 ng; Sigma–Aldrich, Tokyo, Japan) was injected intraperitoneally on the day of immunization. On day 10, each mouse received another intradermal injection of the same mixture of peptides (each 20 μg) emulsified with IFA containing 250 μg of H37RA (Fig. 1A).

2.4. Analysis of cytokine profiles

On day 20 after the first immunization, the spleen was isolated from each $M3R^{+/+}$ and $M3R^{-/-}$ mouse and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. Then, the homogenates were adjusted to 1×10^6 cells/ml and incubated with RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1000 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol, together with a mixture of extracellular peptides (each 5 μg/ml) for 72 h under 5% CO_2 at 37 °C. After collection of the supernatant, the levels of IFN-γ, IL-4 and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) (IFN-γ, IL-4, and IL-17; R&D Systems Minneapolis, MN).

2.5. Adoptive transfer and cell sorting

On day 20 after the first immunization, the spleens were isolated from $M3R^{+/+}$ and $M3R^{-/-}$ mice. The spleens from each group of mice (each $n = 3$) were pooled and homogenized. The red blood cells were removed from the splenocytes by treatment with 0.16 M NH_4Cl solution. After washing with phosphate buffered saline (PBS), the cells were resuspended in PBS.

$CD3^{+}$ T cells were sorted from splenocytes of $M3R^{+/+}$ and $M3R^{-/-}$ by using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The high purity of $CD3^{+}$ T cells (>93%) was confirmed by FACSCalibur (BD PharMingen, San Diego, CA). Cells were resuspended in PBS and 1×10^7 cells were inoculated intravenously into $Rag1^{-/-}$ mice (Fig. 1A).

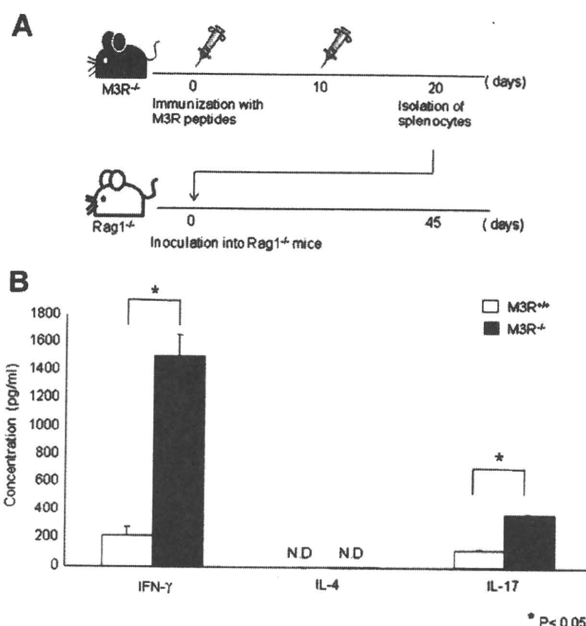

Fig. 1. Cytokine profile of M3R reactive T cells in $M3R^{-/-}$ mice immunized with M3R peptides. (A) $M3R^{-/-}$ and $M3R^{+/+}$ mice were immunized with a murine M3R peptide mixture on days 0 and 10. On the day of immunization, 500 ng of pertussis toxin was injected intraperitoneally. Ten days after booster immunization, the spleens were isolated and transferred into $Rag1^{-/-}$ mice. At day 45 after the transfer, the salivary glands of $Rag1^{-/-}$ recipient mice were analyzed histologically. (B) $M3R^{-/-}$ and $M3R^{+/+}$ mice were immunized followed by a booster dose of M3R peptides mixture emulsified in IFA plus M. tuberculosis. Ten days later, the spleens were isolated from $M3R^{+/+}$ and $M3R^{-/-}$ mice and stimulated with the M3R peptides mixture. The concentrations of IFN-γ, IL-4 and IL-17 in the culture supernatants were measured by enzyme-linked immunosorbent assay. N.D.: not detected. Values are mean ± SD of three independent experiments ($n = 3$ mice per experiment). $*P < 0.05$ (Mann–Whitney U test).

2.6. Measurement of salivary volume

Mice were first anesthetized with intraperitoneal injection of pentobarbital (1.0 mg/kg), then injected subcutaneously with pilocarpine (25 mg/kg). This was followed 15 min later by collection of saliva from the oral cavity using a 200-μl micropipette. The volume of the sample was measured and expressed relative to body weight. Changes in saliva volume were calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (ml)/weight (g)]/[day0 saliva volume (ml)/weight (g)].

2.7. Analysis of antibody levels by enzyme-linked immunosorbent assay

The peptide solution containing all types of extracellular peptides (each 5 μg/ml) was mixed with 0.1 M Na_2CO_3 buffer, pH 9.6, absorbed onto a Nunc-Immunoplate (Nalge Nunc Int, Rochester, NY) at 4 °C overnight, then blocked with 1% bovine serum

albumin (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 1 h at room temperature. Serum at 1:50 dilution in blocking buffer was incubated for 2 h at room temperature. The plates were then washed five times with 0.05% Tween 20 in PBS, and HRP-conjugated anti-mouse IgG antibody diluted 1:1000 in blocking buffer was added for 1 h at room temperature. After washing, 100 μl of TMB solution (Kirkegaard & Perry Laboratories, Inc, Washington, DC) was added as a substrate and then the plates were incubated for 15 min at room temperature in the dark. After adding 50 μl of 1 M phosphate buffer to stop the enzymatic reaction, the optical density was measured at 450 nm by plate spectrophotometry (Bio-Rad Laboratories, Hercules, CA).

2.8. Histological score

The inflammatory lesions were graded histologically (histological sore) using the method proposed by Greenspan et al. [14] as follow: focus score was described as the number of a focus composed of >50 mononuclear cells per 4 mm^2 of tissue. Histological evaluation of the salivary glands was performed in a blind manner, and at least one tissue section from each salivary gland was examined.

2.9. Immunohistochemical analysis

Sections of the salivary glands were thawed, dried, and then fixed with acetone for 10 min. They were incubated with biotin-conjugated anti-mouse CD4, CD8, I-A^b, CD11c (Biolegend, San Diego, CA), rat anti-mouse Thy1 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse B220, CD86, F4/80 (Biolegend) or HRP-conjugated rabbit anti-mouse IgG antibodies (DAKO, Japan) for 2 h. To detect primary antibodies, horseradish peroxidase (HRP)-conjugated streptavidin or HRP-conjugated anti-rat IgG antibodies were added for 30 min. HRP activity was detected using 3,3-diaminobenzidine (DAB; Nichirei, Tokyo) as a substrate. The stained sections were counterstained with Mayes's hematoxylin for 30 s, and mounted with aqueous medium. Control slides were incubated with a dilution buffer containing isotype-matched antibodies instead of the primary antibodies.

Double immunofluorescence staining was performed as follow: Sections of the saliva glands were thawed, dried, and then fixed with 4% paraformaldehyde PBS (Wako) for 10 min. The sections were incubated with rat anti-mouse IFN-γ (Biolegend) and rat anti-mouse IL-17 antibodies (Santa Cruz Biotechnology) for 30 min. To detect primary antibodies, Alexa Fluor 546 goat anti-rat IgG antibody was added for 30 min. The nuclear DNA was stained with DAPI (Sigma–Aldrich).

2.10. Terminal deoxynucleotidyl transferase-mediated nick and labeling staining

Apoptotic cells were detected by *in situ* apoptosis detection kit (Takara Biomedicals, Tokyo). Briefly, frozen sections were fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase/methanol. After washing in PBS, the sections were treated for 5 min on ice with the permeabilisation buffer and then incubated with TdT enzyme reaction for 90 min at 37 °C. An HRP-conjugated anti-FITC antibody was added for 30 min at 37 °C, and the HRP activity was detected using DAB as a substrate. Nuclei were counterstained with hematoxylin.

2.11. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney *U* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. High production of IL-17 and IFN-γ by splenocytes of $M3R^{-/-}$ mice

To compare the immune response to M3R between in $M3R^{-/-}$ and $M3R^{+/+}$ mice, both groups of mice were immunized with the M3R peptide mixture and their splenocytes were cultured with M3R *in vitro* at 20 days after first immunization (Fig. 1A). As shown in Fig. 1B, the production of IL-17 and IFN-γ by splenocytes of immunized $M3R^{-/-}$ mice far exceeded that of $M3R^{+/+}$ mice. On the other hand, IL-4 production was below the detection level in both groups. These results suggest a higher immune response to M3R in $M3R^{-/-}$ than $M3R^{+/+}$ mice.

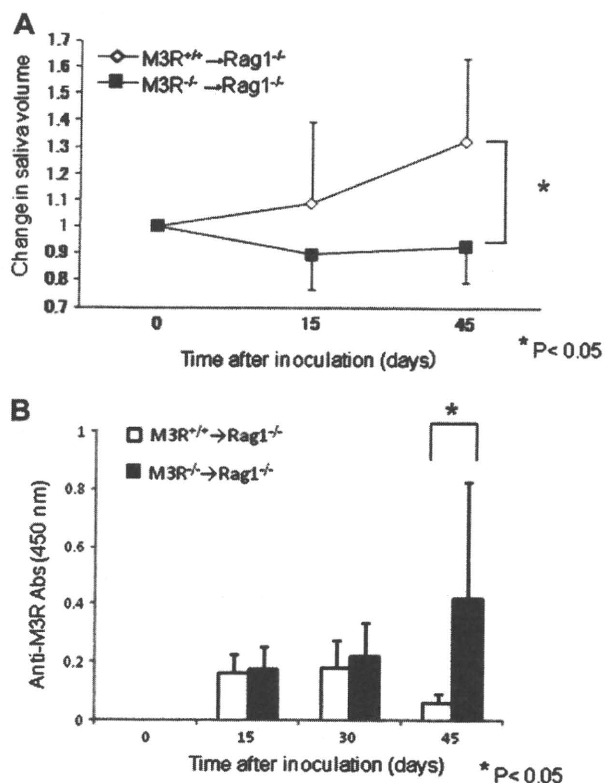

Fig. 2. Reduced salivary secretion and increased levels of anti-M3R antibodies in $M3R^{-/-}$→$Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice inoculated with splenocytes from immunized $M3R^{-/-}$ and $M3R^{+/+}$ mice by intravenous injection into the tail vein. (A) Saliva was collected from $Rag1^{-/-}$ mice at 0, 15, 45 days after the adoptive transfer. The saliva samples were collected from each mouse 15 min after stimulation with pilocarpine. The volume of each saliva sample was measured and adjusted for body weight, and calculated relative to the volume measured at baseline, using the formula [dayN saliva volume (μl)/weight (g)]/[day0 saliva volume (μl)/weight (g)]. (B) Titer of anti-M3R antibodies in sera of $Rag1^{-/-}$ mice obtained at days 0, 15, 30, and 45 after adoptive transfer, determined by ELISA. *$P < 0.05$ versus saliva volume and M3R antibodies derived from $Rag1^{-/-}$ mice inoculated with splenocytes of immunized $M3R^{+/+}$ mice at the same time point, by Mann–Whitney *U* test. Values are mean ± SD of two independent experiments ($n = 6$ mice per experiment).

3.2. Development of sialoadenitis in Rag1$^{-/-}$ mice inoculated with splenocytes from immunized M3R$^{-/-}$ mice

To expose M3R reactive cells to the antigen, we isolated splenocytes from the immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice and inoculated them into M3R-positive Rag1$^{-/-}$ mice (Fig. 1A). First, we examined saliva volume at days 0, 15, and 45 to determine the effect of inoculation of splenocytes of immunized M3R$^{-/-}$ or M3R$^{+/+}$ mice in Rag1$^{-/-}$ mice. The saliva volume decreased gradually from day 15–45 in Rag1$^{-/-}$ mice inoculated with splenocytes of M3R$^{-/-}$ mice compared with those inoculated with splenocytes of M3R$^{+/+}$ mice (Fig. 2A). At day 45 after the cell transfer, serum anti-M3R antibodies were significantly higher in M3R$^{-/-}$→Rag1$^{-/-}$ mice than M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 2B).

To examine the influence of transferred splenocyte from M3R$^{-/-}$ or M3R$^{+/+}$ immunized with the M3R peptides on salivary glands of Rag1$^{-/-}$ mice, M3R$^{-/-}$→Rag1$^{-/-}$ mice and M3R$^{+/+}$→Rag1$^{-/-}$ mice were analyzed histologically. Histological examination of mice at day 45 after inoculation showed marked mononuclear infiltration in the salivary glands and lacrimal glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice but not in M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 3A). However, no inflammatory lesions were observed in the intestine or liver (Fig. 3C). Quantitative analysis of the inflammatory lesions of the salivary glands indicated a higher histological sore for M3R$^{-/-}$→Rag1$^{-/-}$ mice than M3R$^{+/+}$→Rag1$^{-/-}$ mice (Fig. 3B). These results suggested that autoimmune response against M3R-induced infiltration of mononuclear cells in the salivary glands and salivary dysfunction.

To characterize the infiltrating cells in the inflammatory lesions of salivary glands, the frozen sections were analyzed immunohistochemically. The majority of infiltrating cells in M3R$^{-/-}$→Rag1$^{-/-}$ mice were Thy1$^+$ CD4$^+$ T cells, with only few CD8$^+$ T cells. Other cells such as B220$^+$, CD11c$^+$ cells and macrophage formed a minor population and were localized around T cells (Fig. 4A). Furthermore, deposition of IgGs was sometimes noted in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice (Fig. 4A). MHC class II and CD86 were expressed in ductal epithelial cells of the inflammatory lesions of salivary glands from M3R$^{-/-}$→Rag1$^{-/-}$ mice, suggesting that activated epithelial cells acted as APC.

We also examined cytokine production in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ by immunofluorescence staining. The salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice, but not those of M3R$^{+/+}$→Rag1$^{-/-}$ mice, showed differential expression of IFN-γ and IL-17 (Fig. 4B). A few apoptotic cells were noted in the lesions of salivary glands isolated from M3R$^{-/-}$→Rag1$^{-/-}$ mice on TUNEL staining (Fig. 4D), which might explain the salivary destruction and dysfunction.

3.3. M3R reactive T cells and development of sialoadenitis

The above findings indicated that the infiltrated cells in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice were mainly T cells. To confirm that M3R reactive T cells play a role in the development of sialoadenitis in M3R$^{-/-}$→Rag1$^{-/-}$ mice, we transferred purified splenic CD3$^+$ T cells or CD3$^+$ T cells-free splenocytes (CD3$^-$ cells) from M3R$^{-/-}$ mice immunized with M3R peptides mix into Rag1$^{-/-}$ mice (M3R$^{-/-}$ CD3+

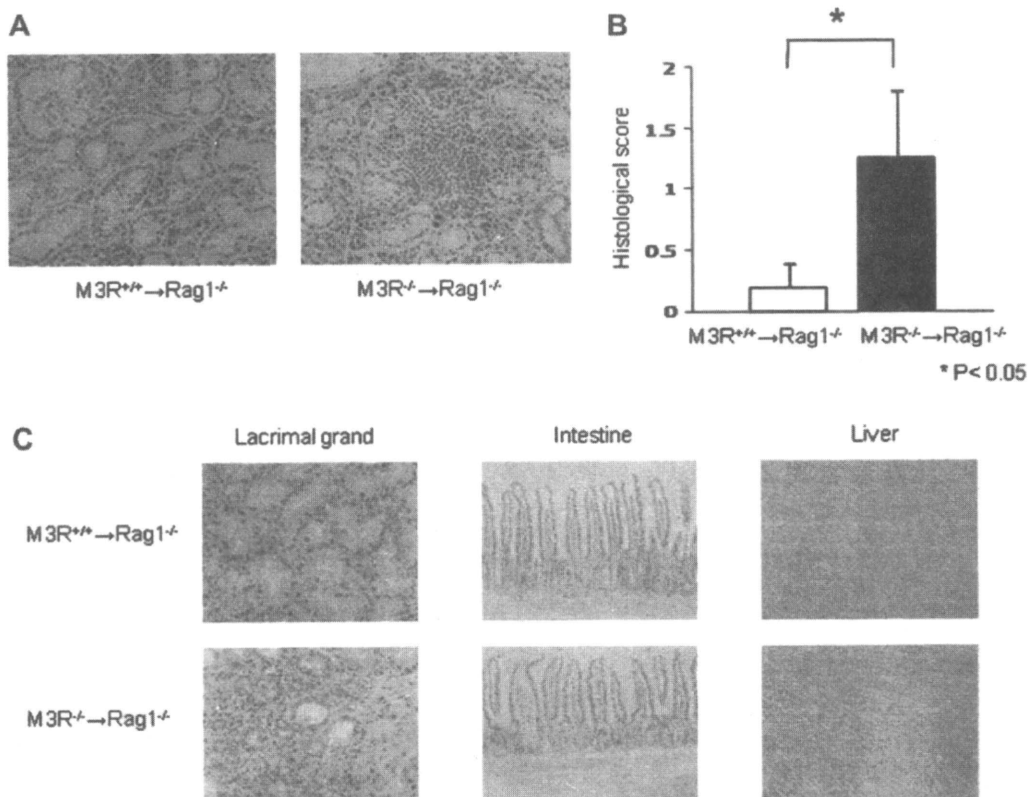

Fig. 3. Infiltration of salivary glands in M3R$^{-/-}$→Rag1$^{-/-}$ mice. Salivary glands isolated from Rag1$^{-/-}$ mice at day 45 after inoculation of splenocytes from immunized M3R$^{-/-}$ and M3R$^{+/+}$ mice. (A) Salivary glands were prepared into 4-μm thick sections, and each section was stained with Mayer's hematoxylin and eosin (H&E). Representative images of five to seven mice in each group. (B) Mean grade (histological score) of inflammatory lesions in salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ and M3R$^{-/-}$→Rag1$^{-/-}$ mice. Values are mean ± SD of five mice. (C) H&E-stained sections of the lacrimal glands, intestine and liver isolated from M3R$^{+/+}$→Rag1$^{-/-}$ mice and M3R$^{-/-}$→Rag1$^{-/-}$ mice. (Original magnification 100×).

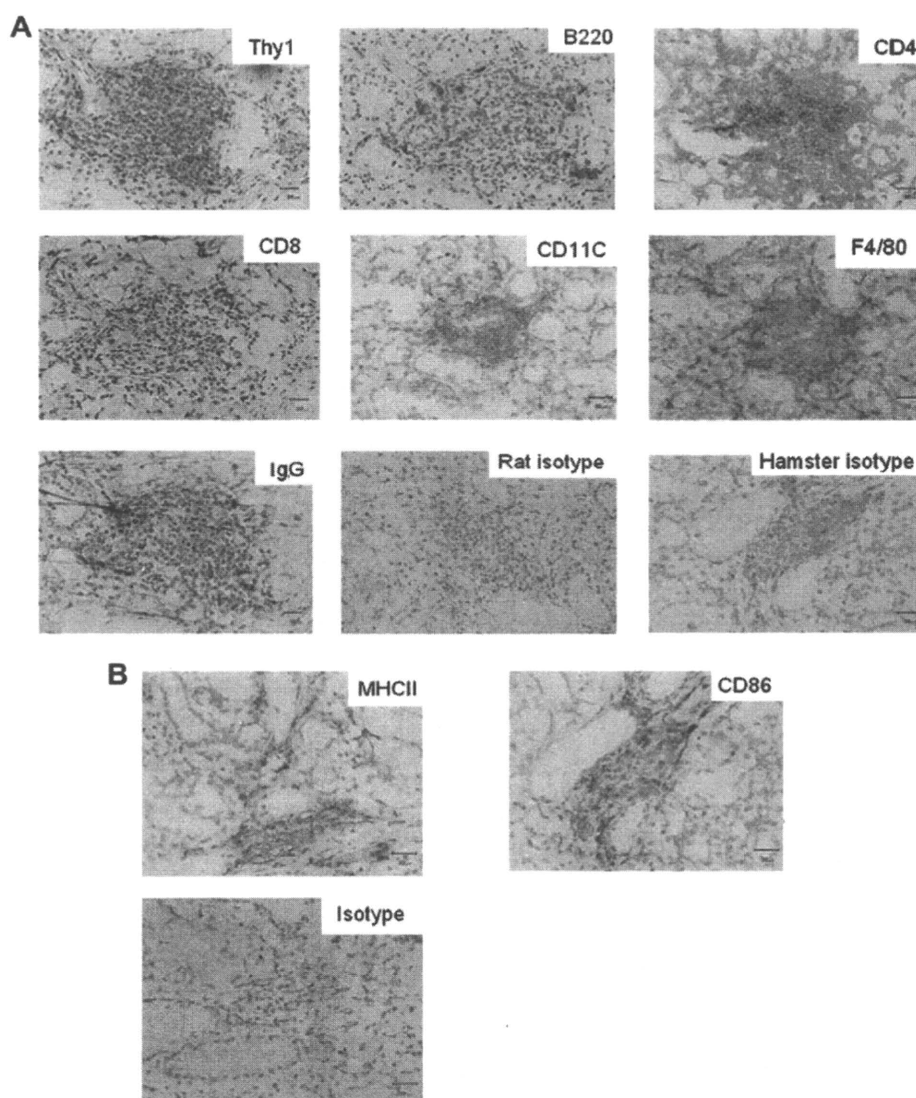

Fig. 4. Histological analysis of salivary glands isolated from M3R$^{-/-}$→Rag1$^{-/-}$ mice. (A) Inflammatory lesions in salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice stained with anti-Thy1, B220, CD4, CD8, CD11c, F4/80 as macrophage specific marker, IgG antibodies and control antibodies. Representative images of five to seven mice. (B) Expression of MHC class II and CD86 detected by immunohistochemistry using anti-I-A^b, CD86 and control antibodies. Representative images of three to five mice. (C) Immunofluorescence analysis of IFN-γ and IL-17 in salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ and M3R$^{-/-}$→Rag1$^{-/-}$ mice. The stained sections were counterstained with H&E, and mounted with aqueous medium. Stained sections were observed at 100× original magnification. Representative images of three to five mice from each group. (D) Apoptotic cells in sections of salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice detected by the TUNEL method. Note the presence of a few apoptotic cells in the salivary glands of M3R$^{+/+}$→Rag1$^{-/-}$ mice. Representative images of three to five mice from each group. Bars = 30 μm.

cells→Rag1$^{-/-}$ mice or M3R$^{-/-}$CD3$^-$ cells→Rag1$^{-/-}$ mice). The purity of CD3$^+$ T cells was 93.6% and CD3$^-$ T cell included only 1.6% of CD3$^+$ T cells (Fig. 5A). Histological analysis showed marked cell infiltration in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice, but not in M3R$^{-/-}$CD3$^-$ cells→Rag1$^{-/-}$ mice (Fig. 5A). The majority of infiltrating cells in the salivary glands of M3R$^{-/-}$ CD3+ cells→Rag1$^{-/-}$ mice were Thy1+ CD4+ T cells, compared with only a few CD8$^+$ T cells (Fig. 5B). These findings suggest that M3R reactive T cells play a crucial role in the pathogenesis of sialoadenitis in the M3R$^{-/-}$→Rag1$^{-/-}$ mice.

4. Discussion

Our previous results (6) demonstrated that 14% of patients with SS harbor autoantibodies against M3R in their sera. In another study, we also reported that peripheral lymphocytes of 4 out of 9 patients with SS included M3R-reactive T cells (12). These results suggested that the underlying pathomechanism of sialoadenitis in patients with SS could perhaps involve the immune response to M3R. The present study is an extension to our previous investigation and was designed to clarify the role of the M3R reactive immune response in the development of SS. For this purpose, M3R$^{-/-}$ mice were immunized with M3R peptides, their splenocytes were transferred into Rag1$^{-/-}$ mice, and the development of SS-like sialoadenitis was examined. Our results showed the development of mild to severe sialoadenitis in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice, indicating the involvement of the M3R reactive immune response in the development of sialoadenitis. The infiltrating cells in the salivary glands of M3R$^{-/-}$→Rag1$^{-/-}$ mice were mainly CD4$^+$ T cells with a few surrounding B cells. The histological findings resemble those of infiltration of lymphocyte subset in the labial salivary glands of

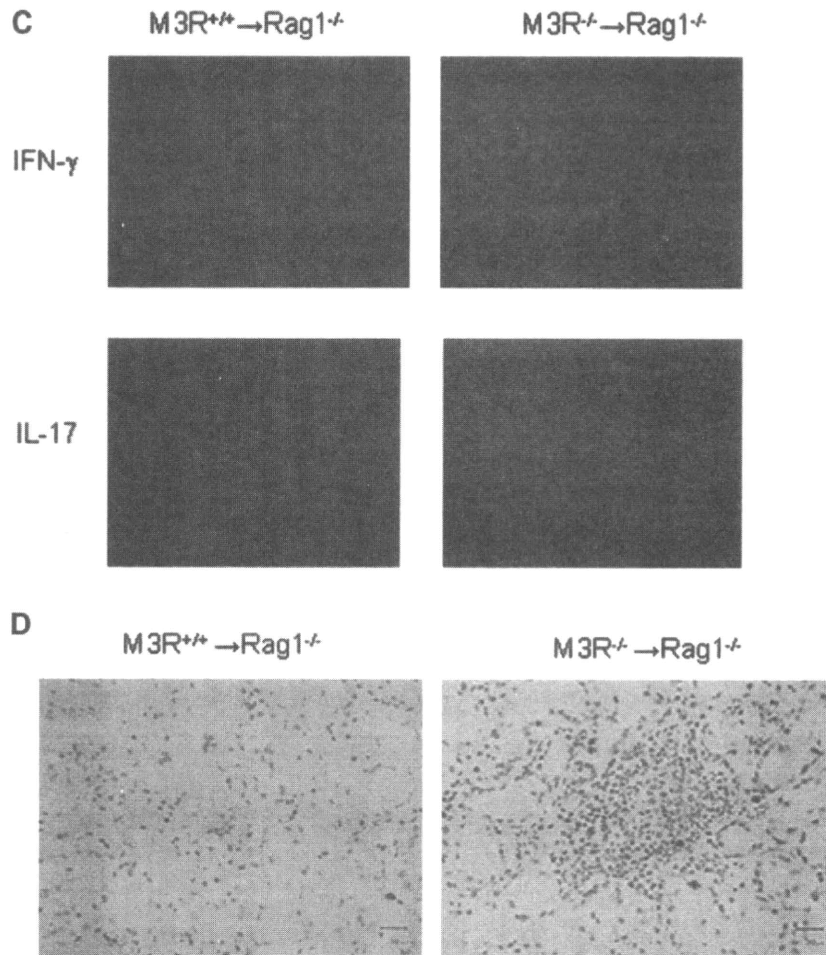

Fig. 4. (*continued*).

patients with SS [15]. Moreover, the $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice had high levels of anti-M3R antibodies and low saliva volume, similar to patients with SS. Previous studies analyzed the levels of anti-M3R antibodies in patients with SS by three methods including peptide-based ELISA, flow cytometry assay and western blot analysis [9,16,17]. Previously, we established peptide-based ELISA for detection of anti-M3R antibodies in SS patients [10,12] and thus applied this method in our mouse model in this study. The results suggest that the M3R molecule acts as an autoantigen to induce an autoimmune reaction in some patients with SS as well as experimental M3R-induced sialoadenitis.

What is the mechanism of M3R-induced sialoadenitis in our mouse model? In $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice, anti-M3R autoantibodies were detected in the serum. IFN-γ– and IL-17-producing cells were also detected in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, the adoptive $CD3^{+}$ T cell transfer experiments showed that $CD3^{+}$ T cells are essential for the development of sialoadenitis in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Thus, M3R reactive T cells are important in the development of antigen-induced sialoadenitis in our mouse model. Although we have no direct evidence in support of a pathogenic role for IFN-γ-producing Th1 cells or IL-17-producing Th17 cells, the latter type of cells might be important in the pathogenesis of M3R-induced sialoadenitis, because none of the IFN-γ dominant T-bet transgenic mice immunized with M3R peptides developed sialoadenitis (data not shown). In this regard, recent studies reported the expression of IL-17 in the salivary glands of patients with SS [18,19]. Thus, further studies using $IFN\text{-}\gamma^{-/-}$ mice or $IL\text{-}17^{-/-}$ mice are necessary.

Why does the combination of $M3R^{-/-}$ mice immunized M3R peptides and $Rag1^{-/-}$ mice enhance the generation of antigen-driven sialoadenitis? It is possible that immunization using a mixture of M3R peptides that includes the N region, and the 1st, 2nd, and 3rd extracellular domains of the M3R molecule, could have induced the immune response against exogenous antigens such as M3R in $M3R^{-/-}$ mice. In fact, M3R reactive T cells and anti-M3R antibodies appeared in $M3R^{-/-}$ mice immunized with M3R peptides. It is possible that these M3R reactive lymphocytes proliferate in $Rag1^{-/-}$ mice and attack the M3R molecule expressed in their own salivary glands, resulting in sialoadenitis.

Why is tolerance to M3R autoantigens broken down in patients with SS? While there is no evidence for the expansion of M3R reactive T cells and B cells in the salivary glands of patients with SS, it is possible that overexpression of M3R autoantigen on APC present in the salivary glands plays a role in the induction of M3R lymphocytes in the peripheral blood with subsequent infiltration of these cells into the tissues [20,21]. The high expression of M3R in the salivary glands may be triggered by previous bacterial or viral infections in the local tissues [22–24].

Based on the results of the present study, we propose that the pathogenesis of sialoadenitis in patients with SS might involve

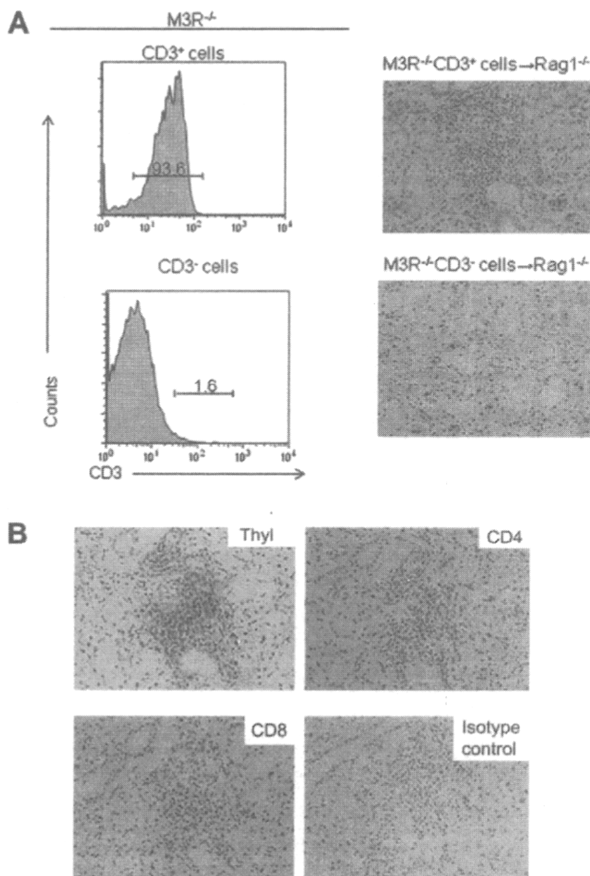

Fig. 5. Role of M3R reactive T cells in the development of sialoadenitis. $CD3^+$ or $CD3^-$ splenocytes of $M3R^{+/+}$ and $M3R^{-/-}$ mice immunized with M3R peptide mix obtained on day 20 after immunization were inoculated into $Rag1^{-/-}$ mice. (A) Salivary glands isolated from $Rag1^{-/-}$ mice at day 45 after transfer of $CD3^+$ or $CD3^-$ cells. Salivary glands were prepared into 4-µm thick sections, and each section was stained with H&E. Representative images of five to seven mice. (B) Sections of salivary glands of $M3R^{-/-}$ $CD3^+$ cells→$Rag1^{-/-}$ mice stained with anti-CD4, CD8, Thy1 and isotype control. Representative images of three to five mice from each group. Magnification 40×.

induction of M3R reactive T cells and B cells in the salivary glands. Importantly, the altered peptide ligands (APL) for M3R binding to the HLA-DR molecule are known to suppress the M3R reactive T cells *in vitro* [25,26]. Therefore, the antigen-induced sialoadenitis in patients with SS could be regulated by APL of M3R. Targeting this antigen-specific modification of autoimmunity in SS could be a potentially useful therapeutic strategy.

In conclusion, we established a new model mouse of sialoadenitis in SS using M3R immunized $M3R^{-/-}$→$Rag1^{-/-}$ mice. Experiments using the new model suggest that the M3R reactive immune response, especially M3R reactive T cells, play a crucial role in the pathogenesis of SS. Targeting this antigen-specific modification of autoimmunity could be a potentially suitable therapeutic strategy in autoimmune diseases such as SS.

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

H. Tsuboi, I. Matsumoto, E. Wakamatsu, Y. Nakamura, M. Iizuka, T. Hayashi, D. Goto, S. Ito and T. Sumida

Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

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Correspondence: Professor T. Sumida, Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-city, Ibaraki 305-8575, Japan.
E-mail: tsumida@md.tsukuba.ac.jp

Summary

M3 muscarinic acetylcholine receptor (M3R) plays a crucial role in the secretion of saliva from salivary glands. It is reported that some patients with Sjögren's syndrome (SS) carried inhibitory autoantibodies against M3R. The purpose of this study is to clarify the epitopes and function of anti-M3R antibodies in SS. We synthesized peptides encoding the extracellular domains of human-M3R including the N-terminal region and the first, second and third extracellular loops. Antibodies against these regions were examined by enzyme-linked immunosorbent assay in sera from 42 SS and 42 healthy controls. For functional analysis, human salivary gland (HSG) cells were pre-incubated with immunoglobulin G (IgG) separated from sera of anti-M3R antibody-positive SS, -negative SS and controls for 12 h. After loading with Fluo-3, HSG cells were stimulated with cevimeline hydrochloride, and intracellular Ca^{2+} concentrations [$(Ca^{2+})i$] were measured. Antibodies to the N-terminal, first, second and third loops were detected in 42·9% (18 of 42), 47·6% (20 of 42), 54·8% (23 of 42) and 45·2% (19 of 42) of SS, while in 4·8% (two of 42), 7·1% (three of 42), 2·4% (one of 42) and 2·4% (one of 42) of controls, respectively. Antibodies to the second loop positive SS-IgG inhibited the increase of $(Ca^{2+})i$ induced by cevimeline hydrochloride. Antibodies to the N-terminal positive SS-IgG and antibodies to the first loop positive SS-IgG enhanced it, while antibodies to the third loop positive SS-IgG showed no effect on $(Ca^{2+})i$ as well as anti-M3R antibody-negative SS-IgG. Our results indicated the presence of several B cell epitopes on M3R in SS. The influence of anti-M3R antibodies on salivary secretion might differ based on these epitopes.

Keywords: autoantibodies, epitopes, function, M3 muscarinic acetylcholine receptor, Sjögren's syndrome

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 pathological 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 and the first, second and third extracellular loops. Among these domains, the second extracellular loop is critical for receptor

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activation by agonists [3]. Therefore, the second extracellular loop of M3R has been the focus of our interest, and we report 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 extracellular loop is the target antigen, the precise epitopes are currently unknown. A recent study reported that the third extracellular loop represents a functional epitope bound by IgG derived from SS patients [6].

The present study was designed to clarify the precise B cell epitopes of M3R and the function of anti-M3R antibodies. For this purpose, we screened sera of SS patients for anti-M3R autoantibodies against all four extracellular domains of M3R by enzyme-linked immunosorbent assay (ELISA) using synthetic peptide antigens and performed functional assays of these antibodies using human salivary gland (HSG) cells. We assessed the correlation between epitopes and function and various clinical features.

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 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 NVSRAAGNFSSPDGTTDDPLGGHT VWQV 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, the sequences of which were FTTYIIMNRWALGNLACDLW for the first extracellular loop, KRTVPPGECFIQFLSEPTITFGTAI for the second and VLVNTFCDSCIPKTFWNLGY for the third (Sigma-Aldrich Japan). As a control peptide, we synthesized a peptide corresponding to the sequences of the third extracellular loop of human-M5 muscarinic acetylcholine receptor (M5R), the sequences of which were STFCDKCVPVTLWH (Sigma-Aldrich Japan). As a negative peptide, we also synthesized a 25-mer peptide whose sequence was SGSGSGSGSGSGSGSGSGSGSGSGS (Sigma-Aldrich Japan).

ELISA

Peptide solution (100 μl/well at 10 μg/ml) in 0·1 M Na_2CO_3 buffer, pH 9·6, was adsorbed onto a Nunc-Immuno plate (Nalge Nunc International, Rochester, NY, USA) overnight at 4°C, and blocked with 5% bovine serum albumin (NSA) (Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline (PBS) for 1 h at 37°C. For the dose-dependent curve, serum from anti-M3R antibodies positive SS and from HC were diluted at 1:25, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600 in blocking buffer, and incubated for 2 h at 37°C. Serum to be examined at 1:50 dilution in blocking buffer was also incubated for 2 h at 37°C. The plates were then washed six times with 0·05% Tween20 in PBS, and 100 μl of solution of alkaline phosphatase-conjugated goat anti-human IgG (Fc; American Qualex, San Clemente, CA, USA) diluted 1:1000 in PBS was added for 1 h at room temperature. After nine washes, 100 μl of p-nitrophenyl phosphate (Sigma) solution was added at a final concentration of 1 mg/ml as alkaline phosphatase substrate. Plates were incubated for 30 min at room temperature in the dark, and the absorbance at 405 nm was measured by plate spectrophotometry. Measurements were performed in triplicate and standardized between experiments by using the absorbance value of the positive control.

Measurement of salivary secretion

We assessed salivary secretion by the gum test. In this test, the volume of saliva is measured after chewing gum for 10 min.

Histopathological examination

Histopathological findings of the labial salivary glands were classified according to Greenspan grading [7].

Expression of M3R mRNA in HSG cells

Total RNA was extracted from HSG cells and cDNA was synthesized by cDNA synthesis kit (Fermentas International, Burlington, Ontario, Canada). Polymerase chain reaction (PCR) was performed with cDNA using the human-M3R-specific primers [2]. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to assess the cDNA yield.

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Expression of M3R proteins on the cell surface of HSG cells

For immunofluorescent analysis, HSG cells were precultured in two-well chamber slides for 48 h. Without fixation, HSG cells were incubated with the first antibodies: anti-human M3R antibody (goat IgG, polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat control IgG (Invitrogen Corporation, Carlsbad, CA, USA) for 2 h. After washing, HSG cells were incubated with the second antibodies: fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG antibodies (IgG; MP Biomedicals, Irvine, CA, USA). Stained HSG cells were observed by fluorescence microscope.

Functional assays

HSG cells (15 000 cells/well) were precultured in 96-well plates for fluorescence assays at 37°C for 48 h. Then, the cells were preincubated with IgG fractions separated from sera of anti-M3R antibodies positive for five SS patients, anti-M3R antibodies negative for one SS patient, and HC by using protein G column (1·0 mg/ml) for 12 h. The referral of the anti-M3R antibodies positive or negative sera was on the basis of our ELISA results. IgG was washed off and the HSG cells were loaded with Fluo-3, which was a fluorescence probe for calcium, for 1 h. Fluo-3 was washed off, and then the HSG cells were analysed. For the Ca^{2+} influx assay, the HSG cells were stimulated with cevimeline hydrochloride, which was a M3R specific agonist at a final concentration of 20 mM. Changes in intracellular calcium concentrations [(Ca^{2+})i] in HSG cells were measured by fluorescence plate reader. Maximum changes of (Ca^{2+})i [peak (Ca^{2+})i – baseline (Ca^{2+})i] in IgG from SS patients or without IgG were shown as ratiometric data compared to maximum change of (Ca^{2+})i in HC [2].

Statistical analysis

Differences between groups were examined for statistical significance using the Mann–Whitney *U*-test, while differences in frequencies were analysed by Fisher's exact probability test. A *P*-value less than 0·05 was considered as the statistically significant difference.

Results

Anti-M3R antibodies in patients with SS and control subjects

The average age of SS patients was 53·1 ± 13·2 years, that of HC was 33·1 ± 8·7 years ($P < 0{\cdot}05$, Mann–Whitney *U*-test). All 42 SS patients were female, 22 of HC female and 20 of HC male. Among 27 patients with secondary SS, 11 were complicated with rheumatoid arthritis (RA), 11 with systemic lupus erythematosus (SLE), two with mixed connective tissue disease (MCTD) and three 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 (Fig. 1a).

Antibodies to the N-terminal region were detected in 42·9% (18 of 42) of SS patients but in only 4·8% (two of 42) of the control ($P < 0{\cdot}05$, Fisher's exact probability test). Antibodies to the first extracellular loop were detected in 47·6% (20 of 42) of SS and 7·1% (three of 42) of the control ($P < 0{\cdot}05$, Fisher's exact probability test). Antibodies to the second extracellular loop were detected in 54·8% (23 of 42) of SS and 2·4% (one of 42) of the control ($P < 0{\cdot}05$, Fisher's exact probability test). Antibodies to the third extracellular loop were detected in 45·2% (19 of 42) of SS and 2·4% (one of 42) of the control ($P < 0{\cdot}05$, Fisher's exact probability test). The frequencies and titres of anti-M3R antibodies against all extracellular domains were significantly higher in SS patients than the control ($P < 0{\cdot}05$, Fisher's exact probability test for frequencies, Mann–Whitney *U*-test for titres) (Fig. 1b).

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, second and third extracellular loops) were detected in one, two, two and one of 28 SS 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 patients (14 of 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 antibody-positive SS (7·3 ± 7·6 years) than -negative SS

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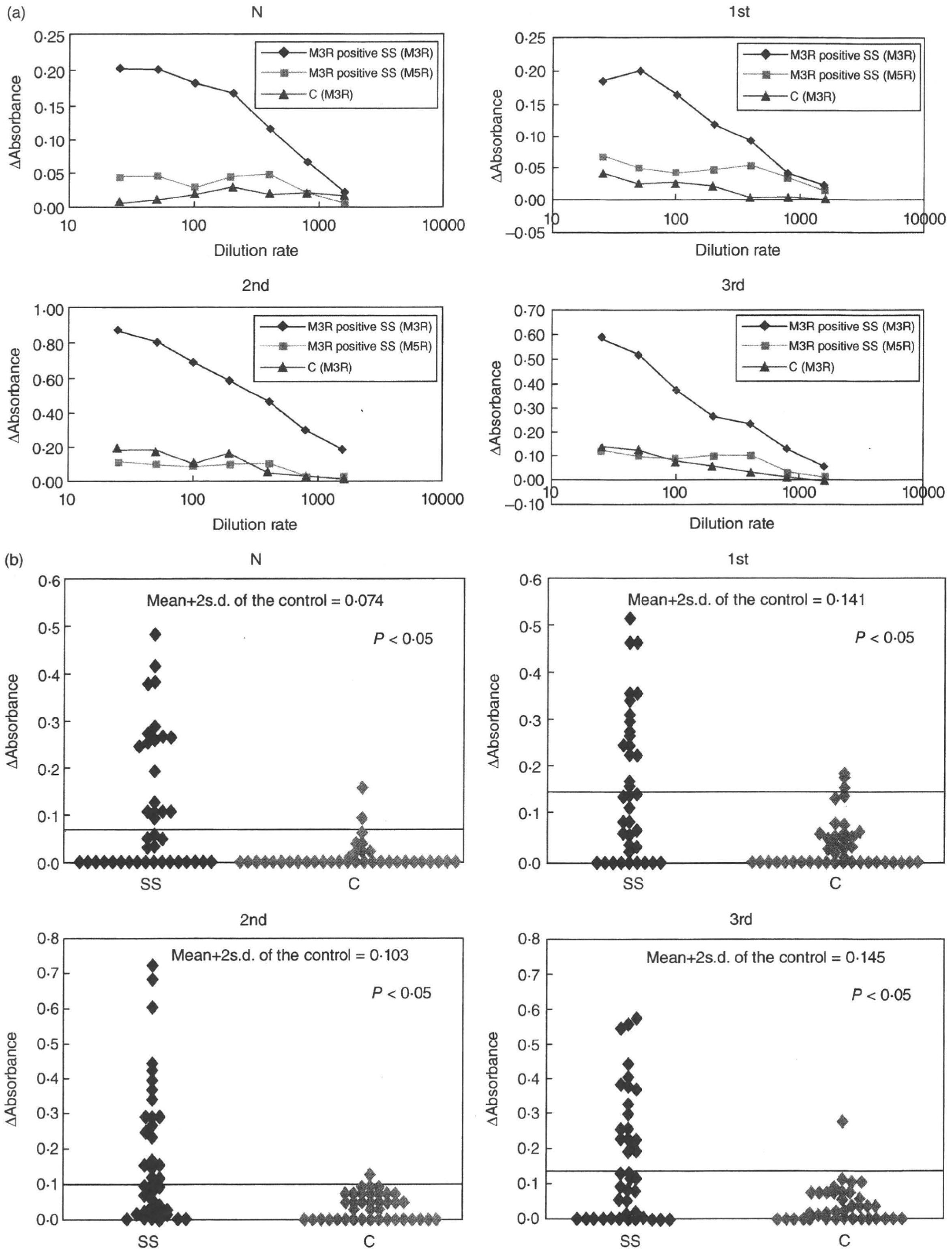

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Fig. 1. (a) The dose-dependent curve on anti-M3 muscarinic acetylcholine receptor (M3R) antibodies. M3R and M5R peptide-specific absorbance values at 405 nm (Δabsorbance) were calculated for each serum sample by subtracting the absorbance value of 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 antibody-positive Sjögren's syndrome (SS), but not in serum from healthy controls (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 antibody-positive SS. (b) Anti-M3R antibodies in patients with SS and control subjects. M3R peptide-specific absorbance values at 405 nm (Δabsorbance) in Sjögren's syndrome (SS) and healthy controls (C). The cut-off level between negative and positive values was the mean ± 2 standard deviation value of the normal control (grey line). The prevalence and titres 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 *U*-test for titres). N: N-terminal region; 1st: first extracellular loop; 2nd: second extracellular loop; 3rd: third extracellular loop.

(15·5 ± 11·1 years, $P < 0{\cdot}05$, Mann–Whitney *U*-test). The positivity for anti-SS-A antibody and the IgG value in serum was more prevalent and higher among anti-M3R antibody-positive SS than -negative SS ($P < 0{\cdot}05$, Fisher's exact probability test and Mann–Whitney *U*-test). In contrast, there were no differences in age, positivity for anti-SS-B antibody and rheumatoid factor, tear volume by Schirmer test, saliva volume by gum test, extra-glandular involvement and Greenspan grading between anti-M3R antibody-positive and -negative SS (Table 2). There is no significant relationship between each B cell epitope and clinical characteristics such as saliva secretion.

Expression of M3R mRNA and proteins in HSG cells

PCR products revealed the expression of M3R mRNA in HSG cells used in the present study. The expected PCR product for M3R was detected at 201 base pairs (bp)

Table 1. B cell epitopes on the M3 muscarinic acetylcholine receptor (M3R).

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

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-M3 muscarinic acetylcholine receptor (M3R) antibody-positive and -negative Sjögren's syndrome (SS) patients.

	Positive SS $n = 28$	Negative SS $n = 28$	*P*-value
Primary/secondary	12/16	3/11	n.s.
Age (years)	51·4 ± 12·1	56·4 ± 12·1	n.s.
Disease duration (years)	7·3 ± 7·6	15·5 ± 11·1	$P < 0{\cdot}05$
Anti-SSA (%)	92·9	57·1	$P < 0{\cdot}05$
Anti-SSB (%)	21·4	14·3	n.s.
Rheumatoid factor (%)	46·4	50·0	n.s.
IgG (mg/dl)	2013 ± 767	1427 ± 515	$P < 0{\cdot}05$
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.

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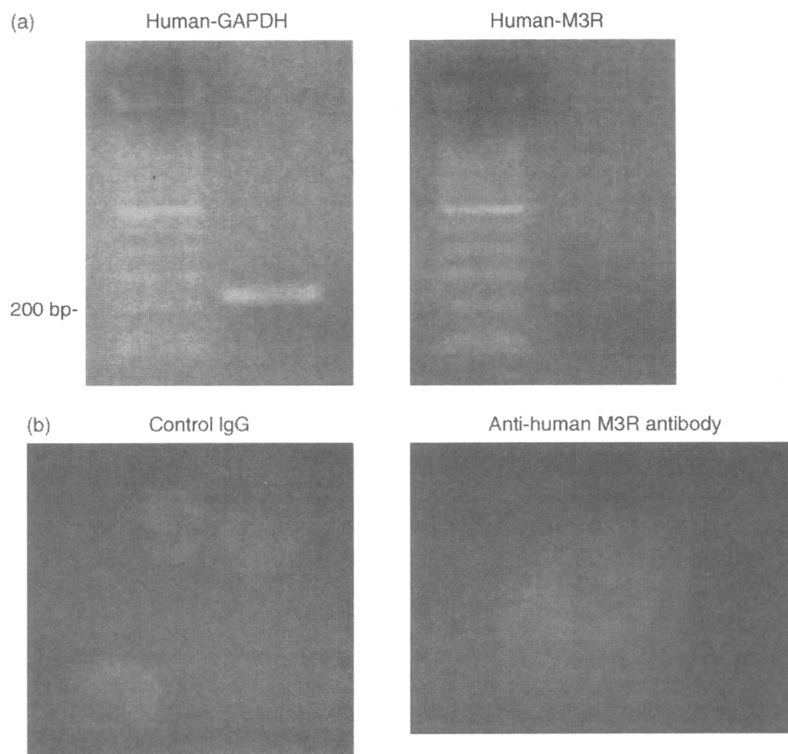

Fig. 2. (a) Expression of M3 muscarinic acetylcholine receptor (M3R) mRNA in human salivary gland (HSG) cells. (b) Expression of M3R proteins on the surface of HSG cells detected by immunofluorescent analysis.

(Fig. 2a). Moreover, M3R proteins were detected on HSG cells stained with anti-human M3R antibody, whereas they were not found with control IgG (Fig. 2b). These results indicated that HSG cells expressed M3R molecules on their surface.

Functional roles of anti-M3R antibodies

IgG derived from two SS patients positive for anti-M3R antibodies to the second extracellular loop inhibited the increase in $(Ca^{2+})i$ induced by cevimeline hydrochloride 16% and 25%, respectively ($P < 0{\cdot}05$, *versus* IgG derived from HC, Mann–Whitney *U*-test) (Figs 3c,d and 4). In contrast, IgG derived from SS patients positive for antibodies to the N-terminal and the first extracellular loop enhanced the increase in $(Ca^{2+})i$ induced by cevimeline hydrochloride 14% and 15%, respectively ($P < 0{\cdot}05$, *versus* IgG derived from HC, Mann–Whitney *U*-test) (Figs 3a,b and 4). IgG derived from a SS patient positive for antibodies to the third extracellular loop had no effect on $(Ca^{2+})I$, as well as IgG derived from an anti-M3R antibody-negative SS patient (Figs 3e and 4).

Discussion

Recently, anti-M3R antibodies have been the focus of interest in rheumatology because of their potential pathogenic role, use as diagnostic markers and being therapeutic targets in patients with SS [1]. Several methods have been used to detect anti-M3R antibodies in SS patients [1]. In functional assays using smooth muscles, IgG fractions from patients with SS (SS-IgG) inhibited carbachol-evoked or nerve-evoked bladder or colon contractions [8,9]. In salivary gland cells, SS-IgG inhibited the rise in $(Ca^{2+})i$ induced by carbachol, and also inhibited pilocarpine-induced AQP5 trafficking to the apical membrane from the cytoplasm [2]. The inhibitory actions of SS-IgG on the rise in $(Ca^{2+})i$ was acutely reversible [10]. Anti-M3R antibodies from SS patients can be detected by immunofluorescent analysis using rat lacrimal glands [11], and by flow cytometry using the M3R-transfected Chinese hamster ovary (CHO) cell line [12]. Moreover, anti-M3R antibodies in sera of SS patients were detected by ELISA using synthetic peptides or recombinant proteins of the second extracellular loop of M3R [13]. We have reported previously the presence of anti-M3R antibodies in a group of patients with SS, which recognized the second extracellular loop by ELISA using synthetic peptides [4,5].

In the present study, we established a standard method to detect anti-M3R antibodies that can be used for screening large patient populations. Functional assays and flow cytometry are too laborious for routine use. Although ELISA is easy, the results from some ELISA systems used for screening anti-M3R antibodies differ widely with regard to the prevalence of anti-M3R antibodies (from 11 to 90%) [4,14]. Furthermore, Cavill *et al.* [15] reported failure to detect anti-M3R antibodies by ELISA using synthetic peptides. In the

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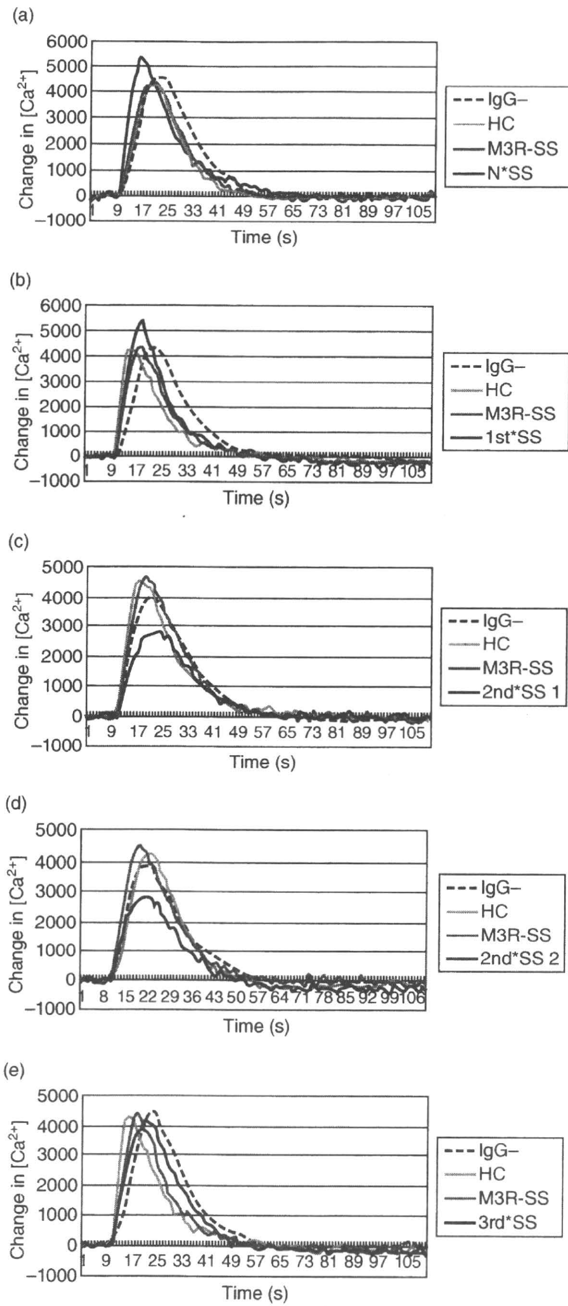

Fig. 3. Functional analysis of anti-M3 muscarinic acetylcholine receptor (M3R) antibodies in Sjögren's syndrome (SS) patients. (a,b) Immunoglobulin G (IgG) derived from SS patient with anti-M3R antibodies to the N-terminal region and the first extracellular loop enhanced the increase in (Ca^{2+})i induced 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. Human salivary gland (HSG) cells were stimulated with cevimeline hydrochloride (20 mM) at 10 s. 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. (c,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; 2nd⁺SS: IgG derived from SS patient positive for anti-M3R antibodies to the second extracellular loop. (e) IgG derived from SS patient positive for antibodies to the third extracellular loop had no effect on the increase in (Ca^{2+})i induced by cevimeline hydrochloride. The traces were representative traces, which were performed in triplicate, and three independent experiments with each IgG; 3rd⁺SS: IgG derived from SS patient positive for antibodies to the third extracellular loop.

present study, we reported higher frequencies and titres of anti-M3R antibodies against all extracellular domains in SS patients than the control. The prevalence of 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 whether or not these linear peptides are really conformational epitopes. However, we showed that 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 extracellular loop did not have an effect on the increase in (Ca^{2+})i, while antibodies against the N-terminal and first extracellular loop enhanced the increase in (Ca^{2+})i. These results

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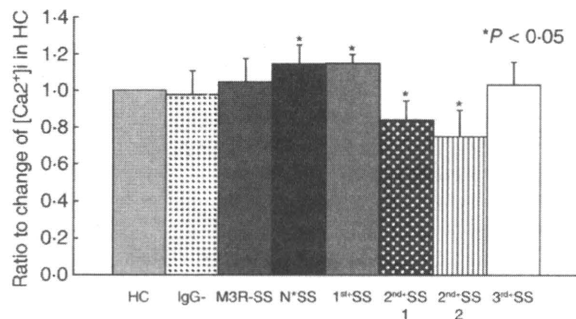

Fig. 4. Summary of B cell epitopes on M3 muscarinic acetylcholine receptor (M3R) and the function of anti-M3R antibodies in Sjögren's syndrome (SS) patients. Mean ± standard deviation values of maximum change in $(Ca^{2+})i$ [peak $(Ca^{2+})i - (Ca^{2+})i$] induced by cevimeline hydrochloride in immunoglobulin G (IgG) from SS patients or without IgG were shown as a ratio compared to maximum change of $(Ca^{2+})i$ in healthy control (HC). The maximum change in HC was described as 1·0. Data were averaged of triplicate and three independent experiments. $^*P < 0·05$ *versus* IgG derived from HC, Mann–Whitney *U*-test, HC, IgG^-, $M3R^-SS$, N^+SS, $1st^+SS$, $2nd^+SS$ and $3rd^+SS$; the same as in Fig. 3.

indicate that the effects of anti-M3R antibodies on the secretion of saliva could be different from these epitopes, although further experiments using antibodies from more patients are necessary.

Although the molecular mechanisms on the difference among individual B cell epitopes have not been elucidated, we could propose the following three possibilities. The first is that antibodies against the second extracellular domain of M3R directly inhibit the intracellular signal pathway, resulting in the decrease of Ca^{2+} influx and reduction of saliva. In contrast, antibodies against N-terminal region and the first extracellular domain of M3R might enhance the intracellular signalling and increase of Ca^{2+} influx. The second is that anti-M3R antibodies binding to the second extracellular domain could inhibit the M3R agonist, and then antibodies suppress indirectly the stimulation of Ca^{2+} influx. The third is that anti-M3R antibodies influence the expression of M3R molecules on HSG. Some antibodies which target the N-terminal region or the first extracellular loop of M3R may be able to up-regulate expression of M3R and enhance Ca^{2+} influx, whereas the other antibodies against the second extracellular domain might down-regulate the expression of M3R on HSG, resulting in a reduction of Ca^{2+} influx. It has been reported that the expression of M3R in salivary glands could be affected by anti-M3R antibodies in patients with SS [1]. Further experiments on the effect of anti-M3R antibodies on M3R signalling, binding to the M3R agonist, and the influence on M3R expression should shed light on the mechanism of the different functions of anti-M3R antibodies.

We have 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 used potentially 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 antibody-positive than in -negative patients with primary SS [14]. Our observations in the present study showed that positivity for anti-SS-A antibody and IgG values in serum was more prevalent and higher in anti-M3R antibody-positive SS patients than -negative SS patients. The disease duration of SS was shorter among anti-M3R antibody-positive SS than -negative SS; however, there was no difference in other clinical and histological features between anti-M3R antibody-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 in particular antibodies against the second extracellular loop of the M3R could suppress the increase in $(Ca^{2+})i$ induced by M3R agonists, resulting in reduction of salivary secretion. Therefore, anti-M3R antibodies might play pathogenic roles in salivary secretion abnormalities characteristic of patients with SS.

Disclosure

None of the authors has any conflict of interest with the subject matter or materials discussed in the manuscript.

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Review

Functional role of M3 muscarinic acetylcholine receptor (M3R) reactive T cells and anti-M3R autoantibodies in patients with Sjögren's syndrome

Takayuki Sumida *, Hiroto Tsuboi, Mana Iizuka, Yumi Nakamura, Isao Matsumoto

Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-city, Ibaraki 305-8575, Japan

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A B S T R A C T

Sjögren's syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration into the lachrymal and salivary glands, leading to dry eyes and mouth. Infiltration is also found in the kidneys, lungs, thyroid, and liver. Immunohistochemical studies have shown that most infiltrating lymphocytes are $CD4^+$ T cell receptor (TCR) $\alpha\beta$ T cells. The antigen specificity of T cells is decided by TCR expressed on T cells. The usage of TCRα and TCRβ genes have been examined by immunological and molecular biological methods. Autoantigens recognized by T cells infiltrating into salivary glands have been analyzed and several candidates for autoantigens have been clarified. In the present study, we focused on M3 muscarinic acetylcholine receptor (M3R) as a salivary gland-specific autoantigen and clarified T cell epitopes and B cell epitopes on M3R. The functions of anti-M3R antibodies and M3R reactive T cells were also carried out. To clarify whether M3R reactive T cells play a crucial role in the generation of autoimmune sialoadenitis, splenic CD3+T cells form $M3R^{-/-}$ mice immunized by M3R peptides were transferred into $Rag\text{-}1^{-/-}$ mice and sialoadenitis analyzed. The functional role of M3R reactive T cells in the generation of SS was also discussed.

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1. TCR repertoire of T cells infiltrating into several organs in patients with Sjögren's syndrome

Previous studies with several polymerase chain reaction (PCR) methods, T cell lines, immunofluorescence staining, or flow cytometry analyses provide evidence about the TCR Vβ and Vα genes on T cells in salivary glands, lachrymal glands, kidneys and peripheral blood from patients with Sjögren's syndrome (SS), suggesting the preferential usage of TCR genes. Moreover, the sequence analysis of the CDR3 region indicates some conserved amino acid motifs. These observations support the notion that infiltrating T cells recognize relatively few epitopes on autoantigen [1–15] as shown in Table 1.

2. Autoantigens recognized by T cells in salivary glands from patients with Sjögren's syndrome

Candidate autoantigens recognized by T cells infiltrating the labial salivary glands of patients with SS have been analyzed, and Ro/SSA 52 kDa, α-amylase, heat shock protein, and TCR BV6 [16–18] have been identified. Recently, we have provided evidence for the presence of M3R reactive T cells in peripheral blood of patients with SS [19].

* Corresponding author. Tel.: +81 29 853 3221; fax: +81 29 853 3222.
E-mail address: tsumida@md.tsukuba.ac.jp (T. Sumida).

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Table 1
TCR repertoire of T cells infiltrating into several organs in Sjögren's syndrome patients.

TCR repertoire	Methods	Authors	Year	Ref.
A) T cells in salivary glands				
Vβ2/Vβ13	Family PCR	Sumida et al.	1992	[1]
Restricted Jβ	Sequencing	Yonaha et al.	1992	[2]
Vβ5,6,13	Anchored PCR	Dwyer et al.	1993	[3]
Restricted Vβ	T cell lines	Legras et al.	1994	[4]
Conserved CDR3	Sequencing	Sumida et al.	1994	[5]
Limited Vα	Inversed PCR	Sumida et al.	1994	[6]
Vβ2,8	IF	Smith etal.	1997	[7]
Fas-sensitive TCR	SSCP	Sumida et al.	1997	[8]
TCR BV2/AV2	Single cell PCR	Matsumoto et al.	1999	[9]
TCR BV13S2	Quantitative PCR	Kay et al.	1999	[10]
B) T cells in lachrymal glands				
Heterogenous Vβ	Family PCR	Mizushima et al.	1995	[11]
Common TCR	SSCP	Matsumoto et al.	1996	[12]
C) T cell in kidneys				
Vβ2	Family PCR	Murata et al.	1995	[13]
D) Peripheral T cells				
Decreased TCR Vβ6.7a	FC	Kay et al.	1991	[14]
TCR BV13.2	ARMS-PCR	Kay et al.	1995	[15]

3. T cell epitopes on M3R in patients with Sjögren's syndrome

The 25mer synthetic amino acids encoding the second extra-cellular domain of M3R (KRTKVPPGECFIAFLSEPTITFGTAI, AA213-237) were used as the antigen for T cells, and the number of IFN-γ producing T cells was counted by flow cytometry using a magnetic activated cell sorting (MACS) secretion assay. The proportion of IFN-γ producing T cells among peripheral blood mononuclear cells (PBMCs) was high in 40% of primary SS patients with HLA-DR B1*0901 allele. The 25mer amino acids contain the anchored motifs that bind to HLA-DR B1*0901, indicating that KRTKVPPGECFIAFLSEPTITFGTAI should be one of T cell epitopes on M3R molecule [19].

4. B cell epitopes on M3R in patients with Sjögren's syndrome

The presence of autoantibodies (Abs) against M3R has been reported, and it is suggested that an immune reaction to M3R plays a crucial part in the generation of SS [20–22]. Robinson et al. [20] demonstrated that human anti-M3R Abs reduce the secretory function in NOD Igμnull mice. Moreover, Bacman et al. [21] clearly showed that human anti-M3R Abs against the second extra-cellular loop of M3R could activate nitric oxide synthetase coupled to the lachrymal gland M3R, suggesting that anti-M3R Abs are a new marker of dry eye SS. The M3Rs are expressed on salivary and lachrymal glands, and thus they should be key receptors involved in the production of saliva and tears after stimulation of acetylcholine. Thence, autoAbs against M3R could interfere with the production of saliva and tears. We analyzed the prevalence of anti-M3R Abs in adult patients with SS and child-onset SS [23,24]. Recently, B cell epitopes recognized by anti-M3R Abs in 42 patients with SS have been examined using synthetic peptides encoding N-terminal region, the first extra-cellular domain, the second extra-cellular domain, and the third extra-cellular domain by ELISA method. Abs to the N-terminal, the first, second and third extra-cellular domains were detected in 42.9% (18/42), 47.6% (20/42), 54.8% (23/42), and 45.2% (19/42) of SS, while in 4.8% (2/42), 7.1% (3/42), 2.4% (1/42), and 2.4% (1/42) of controls, respectively (Tsuboi, et al. personal data). Results were summarized in Table 2. These findings indicated the presence of several B cell epitopes on M3R in SS.

5. Function of anti-M3R Abs in patients with SS

For functional analysis, human salivary gland (HSG) cells were pre-incubated with IgG separated from sera of anti-M3R Abs-positive SS, -negative SS, and controls for 12 h. After loading with Fluo-3, HSG cells were stimulated with cevimeline hydrochloride, and intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) were measured. Abs to the second extra-cellular domain positive SS-IgG inhibited the increase of $[Ca^{2+}]i$ induced by cevimeline hydrochloride. Abs to the N-terminal positive SS-IgG and Abs to the first extra-cellular domain positive SS-IgG enhanced it, while Abs to the third extra-cellular domain positive SS-IgG showed no effect on $[Ca^{2+}]i$ as well as anti-M3R Abs negative SS-IgG (Tsuboi, et al. submitting). The results were shown in Table 2. Our functional data suggested that the influence of anti-M3R Abs on salivary secretion might differ based on these epitopes.

Table 2
B cell epitopes on M3R and function of anti-M3R antibodies in patients with Sjögren's syndrome (SS).

B cell epitopes					
N region	First ECD	Second ECD	Third ECD		
+	+	+	+	50	
+	−	+	+	3.6	
−	+	+	+	3.6	
+	+	−	−	3.6	
+	−	+	−	3.6	
−	+	+	−	7.1	
−	−	+	+	7.1	
+	−	−	−	3.6	Enhancement
−	+	−	−	7.1	Enhancement
−	−	+	−	7.1	Suppression
−	−	−	+	3.6	No effect

ECD, extra-cellular domain.

6. M3R reactive T cells are essential for the generation of autoimmune sialoadenitis

To clarify the role of the immune response to M3R in the pathogenesis of SS, $M3R^{-/-}$ mice were immunized with murine M3R peptides and their splenocytes were inoculated into $Rag1^{-/-}$ ($M3R^{-/-} \rightarrow Rag1^{-/-}$) mice. Anti-M3R Abs were increased in sera and saliva volume was decreased in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Histological examination showed marked infiltration of mononuclear cells in the salivary glands and immunohistochemistry demonstrated that the majority of these cells were $CD4^{+}$ T cells with a few B cells and several IFN-γ- and IL-17-producing cells. Apoptotic cells were present in the salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Moreover, transfer of only $CD3^{+}$ T cells from $M3R^{-/-}$ immunized with M3R peptides into $Rag1^{-/-}$ mice resulted in cell infiltration and destruction of epithelial cells in the salivary glands, suggesting that M3R reactive $CD3^{+}$ T cells play a pathogenic role in the development of autoimmune sialoadenitis (Fig. 1) (Iizuka et al, submitting). Our findings support the notion

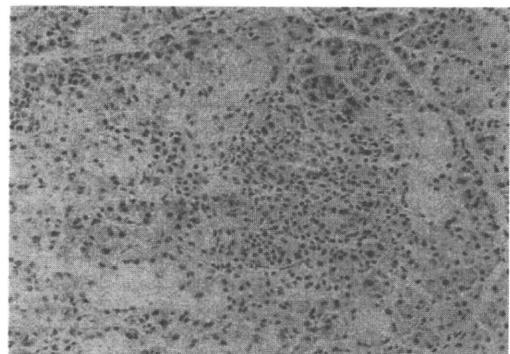

Fig. 1. Histological analysis of salivary glands isolated from $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. Salivary glands isolated from $Rag1^{-/-}$ mice at day 45 after inoculation of splenocytes from immunized $M3R^{-/-}$ and $M3R^{+/+}$ mice. Salivary glands were sectioned at 4 µm, and each section was stained with Mayer's hematoxylin and eosin (H&E).