Table 7 Sensitivities and specificities of the three tested systems for diagnosing SS

	Entire group		Without other CTDs		With other CTDs	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
JPN	79.6	90.4	82.1	90.9	75.3	85.7
AECG	78.6	90.4	83.1	90.9	70.7	85.7
ACR	77.5	83.5	79.1	84.8	74.7	71.4

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

diagnostic systems, as assessed using the kappa coefficient. The data indicate a high level of agreement between the JPN and ACR diagnostic systems (kappa coefficient 0.74), but a low level of agreement between AECG and the other two (kappa coefficient 0.10–0.46) in the diagnosis of all SS, primary SS, and secondary SS.

Discussion

While it is difficult to select the best gold standard system for the diagnosis of CTDs such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and SS, this issue is clinically relevant and important. In SLE, the ACR revised criteria for the classification of SLE (1997) [4] has been adopted for diagnosis in daily clinical practice and for classification purposes in clinical studies. Recently, the Systemic Lupus International Collaborating Clinics (SLICC) has proposed new classification criteria for SLE [5], which has generated interesting discussion about these two criteria among expert rheumatologists. On the other hand, for RA, the 2010 RA classification criteria: an ACR/European League Against Rheumatism (EULAR) collaborative initiative [6] was published recently and is currently used not only in clinical studies for the classification of RA but also in daily clinical practice for the diagnosis of RA. Therefore, these available diagnostic systems for SLE and RA could be regarded as the gold standard for both clinical studies and daily clinical practice. The AECG criteria have been adopted in Western countries for the diagnosis of SS. In Japan, however, both the AECG and JPN criteria are currently being used simultaneously for the classification and diagnosis of SS. On the other hand, the new ACR criteria have been proposed as a uniform classification for SS. At present, there is no gold standard system for the diagnosis of SS in both clinical studies and daily clinical practice, except for expert judgment. This state could create a heterogeneous pool of SS patients, which makes it difficult to analyze the diagnosis, efficacy of treatment, and prognosis of SS patients. Establishing a single set of criteria for SS and selecting a gold standard system for the diagnosis of SS is an important task in Japan.

The present study demonstrated that the sensitivity of the JPN system for all SS and secondary SS, the sensitivity of the AECG system for primary SS, and the specificities of the JPN and AECG systems for all SS, primary SS, and secondary SS were highest among the three systems for diagnosing SS in Japanese patients (relative to clinical judgment as the gold standard). The results also showed high agreement between the JPN and ACR systems, but low agreement between AECG and the other two diagnostic systems for all SS, primary SS, and secondary SS. These results indicate that the JPN and ACR criteria covered similar patient populations, although the sensitivity and specificity were higher for the JPN system than the ACR system. Among the 302 patients with primary SS, 14 did not satisfy the ACR criteria for the diagnosis of SS, although they did meet the criteria of both JPN and AECG. Further analysis of these 14 SS patients also showed that 50 % of these patients had negative pathological findings, 70 % had negative ocular staining, and 50 % were negative for autoantibodies (data not shown). These SS patients could be misdiagnosed by the ACR criteria, resulting in the lower sensitivity of the ACR diagnostic system. On the other hand, among 197 non-SS patients without other CTDs, ten patients satisfied the ACR criteria but not the JPN nor the AECG criteria (data not shown). Further analysis of these ten patients indicated that 80 % were positive for lissamine green ocular staining (Schirmer's test, rose bengal staining, and fluorescein staining were not performed), and 60 % were positive for anti-SS-A antibody (data not shown). Although these patients might be misdiagnosed as primary SS by the ACR criteria, this could not be confirmed because these patients could be positive for other ocular tests adopted by the JPN and AECG diagnostic systems.

The specificities of the criteria for all SS, primary SS, and secondary SS patients used in the JPN and AECG

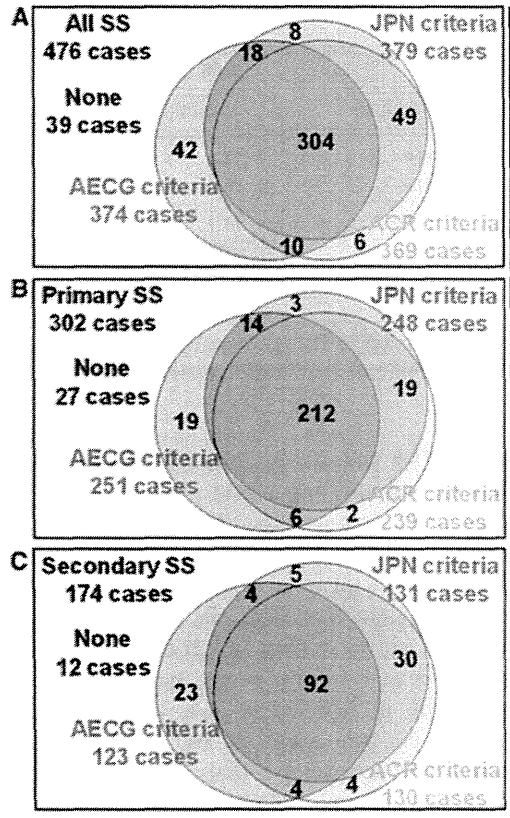

Fig. 1 Venn diagrams showing a comparison of the satisfaction of the three tested systems. **a** Comparison of the satisfaction of the three tested systems, performed using data from all 476 SS patients (302 primary SS and 174 secondary SS). **b** Comparison of the satisfaction of the three tested systems using data on 302 patients with primary SS. **c** Comparison of the satisfaction of the three tested systems using data on 174 patients with secondary SS. *Numbers* show the numbers of patients who satisfied each set of criteria, *None* indicates the number of patients who did not satisfy the criteria of any of the three systems. *JPN criteria* the revised Japanese Ministry of Health criteria for the diagnosis of SS (1999), *AECG criteria* The American-European Consensus Group classification criteria for SS (2002), *ACR criteria* American College of Rheumatology classification criteria for SS (2012)

systems were the same in this study. The reason for the same specificities of the JPN and AECG criteria may be the identical number of non-SS patients (21 patients, including 18 patients without CTDs and 3 patients with CTDs) who satisfied JPN and AECG. However, the JPN and AECG profiles for 20 out of these 21 non-SS patients were completely different, highlighting the low agreement between JPN and AECG, as shown in Table 8.

The sensitivity of AECG for primary SS was highest among the three systems, whereas that of JPN for all SS and secondary SS was highest. Among the 302 primary SS patients, 19 patients only satisfied the AECG criteria. These 19 primary SS patients had high frequencies of dry eye (84.2 %) and dry mouth (100.0 %) but low frequencies of anti-SS-A antibody (10.5 %) and anti-SS-B antibody (0 %). These seronegative primary SS patients with symptoms of dryness could only be diagnosed by the AECG criteria, because only the AECG criteria include symptoms of dryness. This may be the sensitivity of AECG for primary SS was highest among the three systems.

The above findings suggest that JPN provided the best set of criteria necessary for the diagnosis of Japanese patients with SS. Admittedly, however, the results of the present study do not allow us to confirm the superiority of JPN due to the inherent limitations of the study. First, we used the clinical judgment of the physician in charge as the gold standard. In Japan, because the JPN criteria are the criteria used most commonly in daily clinical practice, the clinical judgment could depend on the satisfaction of the JPN criteria. It is better to rely on expert committee consensus based on clinical case scenarios as the gold standard for diagnosis in order to avoid this bias. Second, patients who had been checked for all four criteria of the JPN diagnostic system (pathology, oral, ocular, anti-SS-A/SS-B antibodies) were included in this study, but the methods used for ocular staining varied among the participating institutions. Third, the results of the study could include selection bias. For these reasons, we need a more

Table 8 Agreement among the three tested systems, as assessed using the kappa coefficient

All SS ($n = 476$)	All SS ($n = 476$) (primary SS, $n = 302$, secondary SS, $n = 174$)	Primary SS ($n = 302$)	Secondary SS ($n = 174$)
JPN vs. AECG	0.31	0.46	0.10
JPN vs. ACR	0.74	0.74	0.74
AECG vs. ACR	0.30	0.42	0.12

The "entire group" comprised 694 patients, including 476 with SS (302 patients with primary SS and 174 with secondary SS) and 218 patients with non-SS. The "without other CTDs" group of 499 patients included 302 patients with primary SS and 197 with non-SS. The "with other CTDs" group of 195 patients included 174 patients with secondary SS and 21 with non-SS.

JPN Japanese Ministry of Health criteria for the diagnosis of Sjögren's syndrome (1999), *AECG* The American-European Consensus Group classification criteria for Sjögren's syndrome (2002), *ACR* The American College of Rheumatology classification criteria for Sjögren's syndrome (2012)

sophisticated validation study using randomly selected clinical case scenarios from various institutions and expert committee consensus diagnosis as the golden standard to test the three diagnostic systems for SS, to unify the criteria used for the diagnosis of SS, and ultimately to select the gold standard set of criteria for the diagnosis of SS in Japan.

Currently, the JPN diagnostic system is only used in Japan, because ACR and EULAR have never validated the JPN system. Therefore, we strongly hope that an ACR/EULAR collaborative initiative will validate JPN as well as the AECG and ACR systems.

In conclusion, although this study has a few limitations, the results obtained from it indicate the superiority of the JPN criteria, as it has higher sensitivity and specificity values for the diagnosis of SS in Japanese patients with SS than those of ACR and AECG.

Acknowledgments We thank Dr. F.G. Issa for critically reading the manuscript. This work was supported by Health and Labour Sciences Research Grants for research on intractable diseases (The Research Team for Autoimmune Diseases) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest None.

References

1. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002;61:554–8.
2. Fujibayashi T, Sugai S, Miyasaka N, Hayashi Y, Tsubota K. Revised Japanese criteria for Sjögren's syndrome (1999): availability and validity. Mod Rheumatol. 2004;14:425–34.
3. Shiboski SC, Shiboski CH, Criswell L, Baer A, Challacombe S, Lanfranchi H, et al. American College of Rheumatology classification criteria for Sjögren's syndrome: a data-driven, expert consensus approach in the Sjögren's International Collaborative Clinical Alliance cohort. Arthritis Care Res. 2012;64:475–87.
4. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40:1725.
5. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, Bruce IN, Isenberg D, Wallace DJ, Nived O, Sturfelt G, Ramsey-Goldman R, Bae SC, Hanly JG, Sánchez-Guerrero J, Clarke A, Aranow C, Manzi S, Urowitz M, Gladman D, Kalunian K, Costner M, Werth VP, Zoma A, Bernatsky S, Ruiz-Irastorza G, Khamashta MA, Jacobsen S, Buyon JP, Maddison P, Dooley MA, van Vollenhoven RF, Ginzler E, Stoll T, Peschken C, Jorizzo JL, Callen JP, Lim SS, Fessler BJ, Inanc M, Kamen DL, Rahman A, Steinsson K, Franks AG Jr, Sigler L, Hameed S, Fang H, Pham N, Brey R, Weisman MH, McGwin G Jr, Magder LS. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012;64:2677–86.
6. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62:2569–81.

Mod Rheumatol
DOI 10.1007/s10165-012-0788-5

REVIEW ARTICLE

Anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome

Takayuki Sumida · Hiroto Tsuboi · Mana Iizuka · Hiromitsu Asashima · Isao Matsumoto

Received: 1 October 2012 / Accepted: 17 October 2012

Abstract Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands including salivary and lacrimal glands. Recently, autoantibodies against muscarinic acetylcholine receptor M3 (M3R) have been detected in serum from 9 to 100 % of patients with SS in addition to anti-SS-A and anti-SS-B antibodies. These observations suggest the possibility that anti-M3R antibodies could serve as a new diagnostic test in patients with SS. Some anti-M3R antibodies are directly responsible for salivary underproduction in patients with SS. Thus, strategies designed to eliminate such pathogenic antibodies could help cure SS sufferers. In this review, we summarize the current state of knowledge of anti-M3R autoantibodies in patients with SS and the correlation between B cell epitopes and the function of anti-M3R antibodies.

Keywords Sjögren's syndrome · Autoantibodies · M3 muscarinic acetylcholine receptor · Epitopes · Function

Introduction

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

Recent studies 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+} concentration via inositol 1,4,5-triphosphate (IP3) and IP3 receptors. Consequently, the rise in intracellular Ca^{2+} concentration 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 activation by agonists [3] (Fig. 1). Therefore, the second extracellular loop of M3R has been the focus of interest, and several investigations reported that a subgroup of SS patients have anti-M3R antibodies that recognize 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. Another study reported that the third extracellular loop represents a functional epitope bound by IgG derived from SS patients [6]. In contrast, Tsuboi et al. [7] clarified the precise B cell epitopes of M3R and the function of anti-M3R antibodies in patients with SS. They analyzed sera of SS patients for anti-M3R autoantibodies against all four individual extracellular domains of M3R encoding the N' region, 1st domain, 2nd domain, and 3rd domain by enzyme-linked immunosorbent assay

T. Sumida (✉) · H. Tsuboi · M. Iizuka · H. Asashima · I. Matsumoto
Department of Internal Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsumida@md.tsukuba.ac.jp

Published online: 08 November 2012

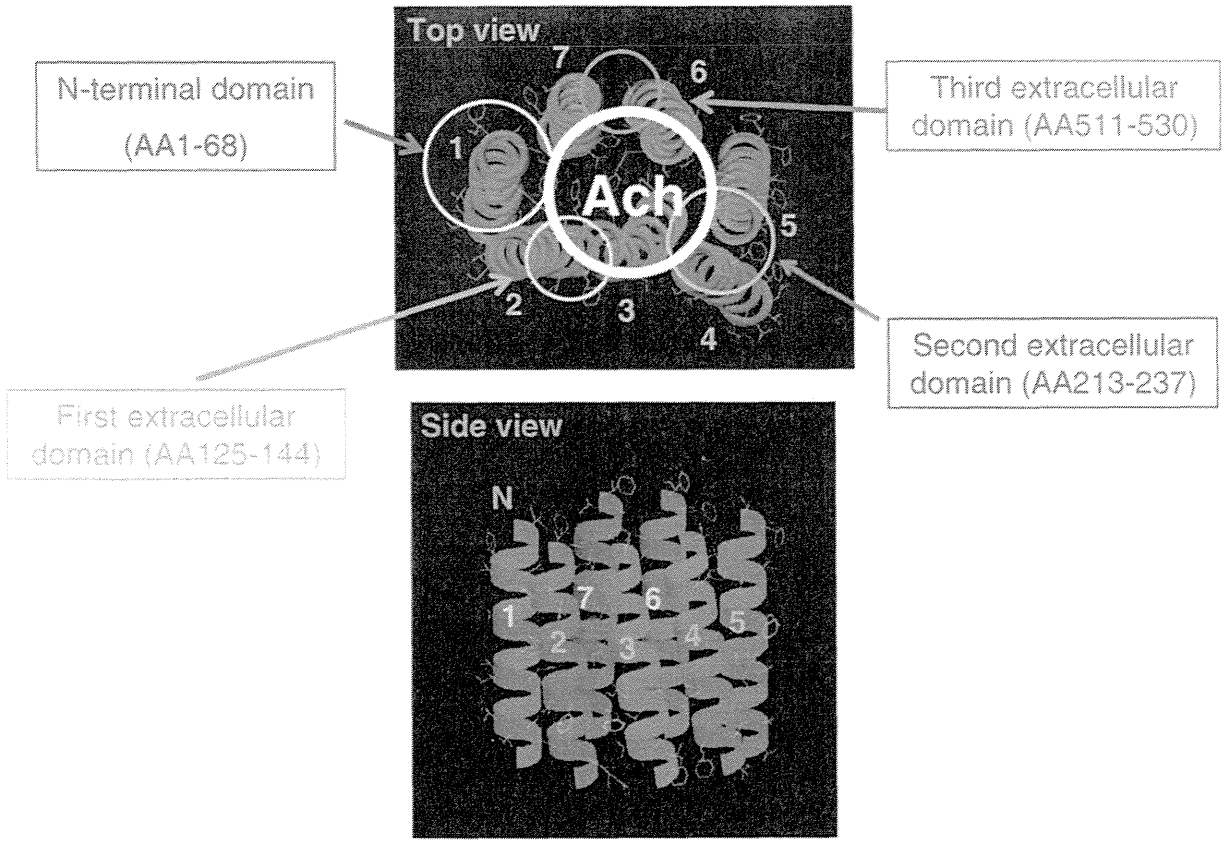

Fig. 1 Structure of M3R molecule and individual domains. The N-terminal domain comprises amino acids 1–68, the first extracellular domain is AA 125–144, the second extracellular domain is AA 213–237, and the third extracellular domain is AA 511–530

(ELISA) using synthetic peptide antigens. Moreover, they performed functional assays of these antibodies using human salivary gland (HSG) cells. In this review, we summarize the current state of knowledge of anti-M3R autoantibodies in patients with SS and the correlation between B cell epitopes and the function of anti-M3R antibodies.

Presence of anti-M3R antibodies in patients with SS (Table 1)

Experimental and clinical evidence point to the presence of anti-M3R antibodies in patients with SS. Bacman and coworkers [8] analyzed IgG in sera of patients with primary SS and then focused on its interaction with M3R in rat exorbital lacrimal glands by indirect immunofluorescence (IF) and binding assay. They showed attenuation of staining for SS IgG in epithelial cells and reported that the strength of staining was weakened by incubation of SS IgG with a synthetic peptide corresponding to the 2nd extracellular loop of M3R. Their results indicated the presence of autoantibodies against the 2nd domain of M3R in patients with SS. Waterman et al. [9] reported that the sera of 5 of 9 (55 %) patients with primary SS and from 6 of 6 patients (100 %) with secondary SS induced approximately 50 % inhibition of carbachol-induced bladder contraction. Furthermore, Gao et al. [10] generated human M3R-expressing CHO cells. They also detected anti-M3R autoantibodies in 9 of their 11 (82 %) SS patients, of the IgG1, IgG3, and IgA isotypes. Naito et al. [4] also detected autoantibodies against the 2nd extracellular domain of M3R in about 9 % (11/122) of their patients with primary SS using synthetic peptides encoding M3R AA213–237. They also detected antibodies against the 2nd extracellular domain of M3R (AA213–237) in 14/102 (14 %) of their patients with secondary SS. Their results were the first report on the binding of anti-M3R antibodies against synthetic peptides. Marczinovits et al. [11, 12] detected the antibodies against a 16-mer synthetic peptide KRTVPPGECFIQFLSE (KRSE 213–228) and recombinant glutathione *S*-transferase (GST)–KRSE fusion protein. The sensitivities of the assays used in their study were 77.5–90 % for KRSE and 97 % for GST–KRSE, and immunological recognition with the recombinant fusion antigen was significantly better than that for the free peptide. Nakamura

Table 1 Presence of anti-M3R Abs in patients with SS

SS	B cell epitopes	Frequency (%)	Method	Year	References
Primary SS IgG	2nd		IF (rat LG)	1998	[11]
Primary SS IgG	M3R on bladder	55	Functional assay	2000	[9]
Secondary SS IgG	M3R on bladder	100	Functional assay	2000	[9]
IgG1, IgG3, IgA	M3R	82	M3R-CHO	2004	[10]
Primary SS	2nd (213–237)	9	ELISA	2005	[4]
Secondary SS	2nd (213–237)	14	ELISA	2005	[4]
Primary SS	2nd (213–228)	77.5–90	ELISA	2005	[11, 12]
	2nd (fusion protein)	97	ELISA	2005	[11]
Juvenile-onset SS	2nd (213–237)	52.6	ELISA	2008	[5]
Primary + secondary SS	N-terminal (1–68)	42.9	ELISA	2010	[7]
	1st domain (125–144)	47.6	ELISA	2010	[7]
	2nd domain (213–237)	54.8	ELISA	2010	[7]
	3rd domain (511–530)	45.2	ELISA	2010	[7]
Primary SS	Cycle-2M3R (205–220)	62.2	ELISA	2011	[13]

LG lacrimal gland

et al. [5] showed high prevalence (52.8 %, 20/38) of autoantibodies to synthetic peptides encoding the 2nd extracellular domain of M3R in patients with juvenile-onset SS, suggesting that anti-M3R Abs could be useful as a diagnostic marker for juvenile-onset SS.

Tsuboi et al. [7] examined the prevalence of autoantibodies against each of four synthetic peptides of M3R in patients with SS. They found antibodies to the N-terminal, 1st, 2nd, and 3rd domains of SS in 42.9 % (18 of 42), 47.6 % (20 of 42), 54.8 % (23 of 42), and 45.2 % (19 of 42), compared with 4.8, 7.1, 2.4, and 2.4 % of healthy subjects. These findings confirm the presence of autoantibodies against not only the 2nd extracellular domain of M3R but also the N-terminal, 1st, and 3rd extracellular domains of M3R in sera of patients with SS, and suggest that their detection could be employed for diagnosis of SS.

Recently, He et al. [13] reported the presence of antibodies against cycle 2nd extracellular domain of M3R (AA205–220) (anti-c2M3RP) in the sera of 62.2 % of their patients with primary SS. The specificity of anti-c2M3RP antibodies was 95.1 % and much higher than that for linear polypeptide (84.7 %), suggesting that c2M3RP might act as an autoantigen and play a role in the production of anti-c2M3RP antibodies.

Although it is not know why the positive rates of anti-M3R Abs in patients with SS vary among the past reports (from 9 to 100 %), we can speculate the following possibilities: The first possibility is the antigenic difference between proteins on cellular membrane [9], fusion proteins [11], and the synthetic amino acids [4, 5, 7, 10–13]. The second is the quality of purification of synthetic amino acids using for antigen in ELISA assay [4, 5, 7, 10–13]. Therefore, it is necessary to establish a standard assay system to detect anti-M3R Abs in serum in the near future.

The frequency of anti-M3R Abs in serum from patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) was 1 % (1/105) and 0 % (0/97), respectively [4]. Thus, we concluded that anti-M3R Abs might be specifically present in patients with SS, suggesting a diagnostic marker in the subgroup of SS.

B cell epitopes on M3R and functional analysis of anti-M3R antibodies (Table 2)

Functional analysis of IgG in SS patients was conducted by Bacman et al. [8] using nitric oxide synthase (NOS) activation and cyclic guanosine monophosphate (cGMP) production in rat exorbital lacrimal glands. They demonstrated that antibodies against the 2nd extracellular domain of M3R suppressed both NOS activation and cGMP production, suggesting that chronic interaction of anti-M3R antibodies on lacrimal glands could lead to tissue damage through NO release after immunological stimulation. In another study, Waterman et al. [9] demonstrated that their IgG fraction purified from the sera of SS patients inhibited the action of carbachol-induced acetylcholine at M3R in neuronal cells, indicating that the IgG fraction from SS patients had antimuscarinic receptor activity. Cavill et al. [14] purified anti-M3R antibodies using affinity column and tested their concentration-dependent inhibition of carbachol-induced colon contractions. They demonstrated that anti-M3R

Table 2 B cell epitopes on M3R and function of anti-M3R antibodies in patients with SS

SS	B cell epitopes	Function	Method	Year	References
Primary SS IgG	2nd	Decrease	NOS/cGMP (rat LG)	1998	[8]
Primary + secondary SS IgG	M3R	Inhibition	Carbachol/Ach-stimulated bladder contractions	2000	[9]
M3R purified IgG	2nd	Inhibition	Carbachol-evoked colon contraction	2004	[14]
IgG	M3R	Inhibition	Pilocarpine-induced Ca influx AQP-5 trafficking (rat PAC)	2004	[2]
Primary SS	2nd (213–228)		Associated with leukopenia	2005	[12]
Primary SS IgG	M3R	Reduction	Ca influx (mouse and human SAC)	2006	[15]
Primary + secondary SS IgG	3rd (514–527)	Inhibition	Ca influx (HSG)	2008	[6]
Primary + secondary SS	N-terminal (1–68)	Increase	Ca influx (HSG)	2010	[7]
	1st (125–144)	Increase	Ca influx (HSG)	2010	[7]
	2nd (213–237)	Decrease	Ca influx (HSG)	2010	[7]
	3rd (511–530)	No change	Ca influx (HSG)	2010	[7]
Mouse monoclonal M3R Abs	2nd (213–237)	Decrease	Ca influx (HSG)	2012	[16]
Primary SS IgG	2nd (M3RP205–220)		Link to saliva flow rate	2012	[13]
Primary SS IgG	M3R		M3R internalization (HSG)	2012	[17]

PAC parotid acinar cells, *SAC* submandibular acinar cells, *HSG* human salivary gland cell lines, *Ach* acetylcholine

antibodies from SS patients inhibited colon contractions, supporting the notion that antibodies against the 2nd extracellular domain of M3R share functional properties in patients with SS. In a study on another tissue, Li et al. [2] purified the IgG fraction from sera of SS patients and analyzed its inhibitory activity on pilocarpine-induced Ca influx and AQP-5 expression. Using rat parotid acinar cells, they showed that SS IgG inhibited Ca influx and modulated pilocarpine-induced AQP-5 trafficking to the apical membrane, suggesting that anti-M3R antibodies are associated with glandular dysfunction and impaired autonomic function in SS patients. Dawson et al. [15] examined the function and activity of IgG isolated from patients with primary SS by using microfluorimetric Ca^{2+} imaging and patch-clamp electrophysiological techniques in mouse and human submandibular acinar cells. They demonstrated that anti-M3R antibodies abrogated carbachol-induced increase in Ca^{2+} in mouse and human acinar cells by approximately 50 %, indicating that IgG from SS patients contains autoantibodies that can reduce saliva production.

Koo et al. [6] analyzed the role of antibodies using microspectrofluorometry and a surface plasmon resonance-based optical biosensor system (BIAcore system). They showed that antibodies against the 3rd extracellular domain of M3R had inhibitory activity against carbachol-induced Ca^{2+} influx in human salivary gland cell lines. They proposed that the 3rd extracellular loop of M3R represented a functional epitope bound by SS IgG. In contrast, Tsuboi et al. [7] demonstrated that only autoantibodies against the 2nd extracellular domain of M3R suppressed cevimeline-HCl-induced Ca^{2+} influx in human salivary gland cell lines, whereas antibodies against the N-terminal and 1st extracellular domain of M3R enhanced Ca^{2+} influx while anti-3rd extracellular domain of M3R had no activity. These findings were supported by two newly established murine monoclonal antibodies against the human 2nd extracellular domain of M3R (213–237) [16]. These two monoclonal antibodies significantly suppressed cevimeline-HCl-induced Ca^{2+} influx in human salivary glands. Moreover, He et al. [13] provided clinical evidence for the close association of anti-M3RP205–220 (2nd extracellular domain of M3R, AA205–220) IgG from SS patients with salivary flow rate, suggesting that these anti-M3R antibodies may be functional. Kovacs et al. [12] also reported that autoantibodies to the second extracellular domain of M3R (AA213–228) were associated with leukopenia in patients with SS. Further examination using specific monoclonal antibodies against each of the four domains is needed to clarify the importance of B cell epitope in dysfunctional salivary production.

Recently, Jin et al. [17] reported that IgG from patients with primary SS reduced the expression level of M3R in the membrane, inhibited carbachol-induced Ca^{2+} transients in human salivary gland cells, and decreased membrane clathrin expression. These results suggest that SS IgG induces internalization of M3R partly through a clathrin-

mediated pathway. They also provide support to the notion that anti-M3R antibodies cause salivary dysfunction via not only reduction in Ca^{2+} influx but also downregulation of M3R molecules on the epithelial cells of salivary glands of patients with SS.

Conclusions

This review summarizes the prevalence of autoantibodies against M3R in patients with SS and analyzes the correlation between B cell epitopes and the function of anti-M3R antibodies. Although there remain several issues that need to be resolved, we can conclude that detection of anti-M3R antibodies present in peripheral circulation can serve as a new diagnostic test in patients with SS. The availability of assays that simplify the detection of anti-M3R antibodies helps in establishing the diagnosis of SS. Moreover, since certain anti-M3R antibodies are directly responsible for salivary underproduction in patients with SS, strategies designed to eliminate such pathogenic antibodies could help cure SS sufferers.

Acknowledgments The authors thank Dr. F.G. Issa for critical reading of the manuscript. This work was supported by the Research Program for Intractable Diseases, Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare, Japan and the Ministry of Education, Culture, Sports, Science, and Technology.

Conflict of interest None.

References

1. Dawson L, Tobin A, Smith P, Gorton T. Antimuscarinic antibodies in Sjögren's syndrome. Arthr Rheum. 2005;52:2984–95.
2. Li J, Ha YM, Ku NY, et al. Inhibitory effects of autoantibodies on the muscarinic receptors in Sjögren's syndrome. Lab Invest. 2004;84:1430–8.
3. Scarselli M, Li B, Kim SK. Wess. Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. J Biol Chem. 2007;282:7385–96.
4. Naito Y, Matsumoto I, Wakamatsu E, et al. Muscarinic acetylcholine receptor autoantibodies in patients with Sjögren's syndrome. Ann Rheum Dis. 2005;64:510–1.
5. Nakamura Y, Wakamatsu E, Matsumoto I, et al. High prevalence of autoantibodies to muscarinic-3 acetylcholine receptor in patients with juvenile-onset Sjögren's syndrome. Ann Rheum Dis. 2008;67:136–7.
6. Koo NY, Li J, Hwang SM, et al. Functional epitopes of muscarinic type 3 receptor which interacts with autoantibodies from Sjögren's syndrome patients. Rheumatology. 2008;47:828–33.
7. Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, Goto D, Ito S, Sumida T. New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjogren's syndrome. Clin Exp Immunol. 2010;162: 53–61.
8. Bacman SR, Berra A, Sterin-Borda L, Borda ES. Human primary Sjögren's syndrome autoantibodies as mediators of nitric oxide release coupled to lacrimal gland muscarinic acetylcholine receptors. Curr Eye Res. 1998;17:1135–42.
9. Waterman SA, Gordon TP, Rischmueller M. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. Arthr Rheum. 2000;43: 1647–54.
10. Gao J, Cha S, Jonsson R, Opalko J, Peck AB. Detection of anti-type 3 muscarinic acetylcholine receptor antibodies in the sera of Sjögren's syndrome patients by use of a transfected cell line assay. Arthr Rheum. 2004;50:2615–21.
11. Marczinovits I, Kovacs L, Gyorgy A, Toth GK, Dorgai L, Molnar J, Pokorny G. A peptide of human muscarinic acetylcholine receptor 3 is antigenic in primary Sjögren's syndrome. J Autoimmun. 2005;24:47–54.
12. Kovacs L, Marczinovits I, Gyorgy A, Toth GK, Dorgai L, Pal J, Molnar J, Pokorny G. Clinical associations of autoantibodies to human muscarinic acetylcholine receptor 3213–228 in primary Sjögren's syndrome. Rheumatology. 2005;44:1021–5.
13. He J, Qiang L, Ding Y, Wei P, Li YN, Hua H, Li ZG. The role of muscarinic acetylcholine receptor type 3 polypeptide (M3RP205–220) antibody in the saliva of patients with primary Sjogren's syndrome. Clin Exp Rheumatol. 2012;30:322–6.
14. Cavill D, Waterman SA, Gordon TP. Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjögren's syndrome. Scand J Immunol. 2004;59:261–6.
15. Dawson LJ, Stanbury J, Venn N, Hasdimir B, Rogers SN, Smith PM. Antimuscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. Arthr Rheum. 2006;54: 1165–73.
16. Tsuboi H, Nakamura Y, Iizuka M, Matsuo N, Matsumoto I, Sumida T. Generation and functional analysis of monoclonal antibodies against the second extracellular loop of human M3 muscarinic acetylcholine receptor. Mod Rheumatol. 2012;22:264–71.
17. Jin M, Hwang S-M, Davies AJ, Shin Y, Bae J-S, Lee J-H, Lee EB, Song YW, Park K. Autoantibodies in primary Sjogren's syndrome patients induce internalization of muscarinic type 3 receptors. Biochim Biophys Acta. 2012;1822:161–7.

Mod Rheumatol
DOI 10.1007/s10165-012-0785-8

RAPID COMMUNICATION

The crucial roles of IFN-γ in the development of M3 muscarinic acetylcholine receptor induced Sjögren's syndrome-like sialadenitis

Mana Iizuka · Hiroto Tsuboi · Naomi Matsuo · Yuya Kondo · Hiromitsu Asashima · Minoru Matsui · Isao Matsumoto · Takayuki Sumida

Received: 20 July 2012 / Accepted: 10 October 2012

Keywords Sjögren's syndrome · M3 muscarinic acetylcholine receptor · IFN-γ · Apoptosis · Sialadenitis

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. Auto-antigens recognized by T cells infiltrating the salivary glands of patients with SS have been analyzed, and several candidate auto-antigens such as M3 muscarinic acetylcholine receptor (M3R) have been identified. The presence and specificity of anti-M3R antibodies in patients with SS have been examined [1–3]. We also reported the presence of IFN-γ-producing M3R-reactive $CD4^+$ T cells in 40 % of SS patients with SS [4]. Several studies also detected high levels of IFN-γ in the salivary glands of SS patients, and then enhanced activity of T cells, B cells, and macrophages, resulting in the destruction and dysfunction of tissue glands [5, 6]. In contrast, IL-17-producing T cells were also found in salivary glands from patients with SS [7].

Our previous study showed that M3R-reactive T cells were involved in the pathogenesis of sialadenitis using M3R-induced sialadenitis (MIS) mice, which are thought to be model mice for SS. In MIS mice, $CD3^+$ T cells were essential for the generation of sialadenitis. Moreover, both IFN-γ and IL-17 were produced by M3R-reactive T cells and were detected in salivary glands, whereas neither IFN-γ nor IL-17 was detected in the sera [8]. However, we have no evidence that the cytokines INF-γ and/or IL-17 are important in the development of sialadenitis. In the present study, to address the question of whether IFN-γ is important in the development of sialadenitis, we generated $M3R^{-/-}\times$IFN-$\gamma^{-/-}$ mice, immunized with M3R peptides, and transferred their splenic cells to Rag-$1^{-/-}$ mice.

Histological findings showed that sialadenitis was more severe in $M3R^{-/-}\times$IFN-$\gamma^{-/-} \rightarrow Rag1^{-/-}$ than that in $M3R^{+/+} \rightarrow Rag1^{-/-}$ mice, but milder than that in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice (Fig. 1a). Quantitative analysis using histological scores indicated that mononuclear cell infiltration was significantly increased in $M3R^{-/-}\times$IFN-$\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice compared with that in $M3R^{+/+} \rightarrow Rag1^{-/-}$ mice ($P < 0.05$), but significantly decreased compared with that in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice ($P < 0.05$) (Fig. 1b). These observations support the notion that IFN-γ might play a crucial role in the generation of SS-like sialadenitis. The absence of IFN-γ- and presence of IL-17-producing cells in the salivary glands of $M3R^{-/-}\times$IFN-$\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice were verified by immunohistochemical staining (Fig. 1c). IL-17-producing cells in inflammatory lesions were identified in both $M3R^{-/-}\times$IFN-$\gamma^{-/-} \rightarrow Rag1^{-/-}$ and $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice. IFN-γ and IL-17 were not detected in sera from $M3R^{-/-}\times$IFN-$\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice, nor in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice (data not shown). In $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice, the expression of IL-17 was also observed in salivary glands, as was IFN-γ expression. As we have no direct evidence in support of a pathogenic role of IL-17 in MIS, further studies using $M3R^{-/-}\times$IL-$17^{-/-}$ mice will be necessary to clarify the function of IL-17-producing M3R-reactive T cells.

I. Iizuka · H. Tsuboi · N. Matsuo · Y. Kondo · H. Asashima · I. Matsumoto · T. Sumida (✉)
Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tenodai, Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsumida@md.tsukuba.ac.jp

M. Matsui
Departments of Clinical Research and General Medicine, Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan

Published online: 26 October 2012

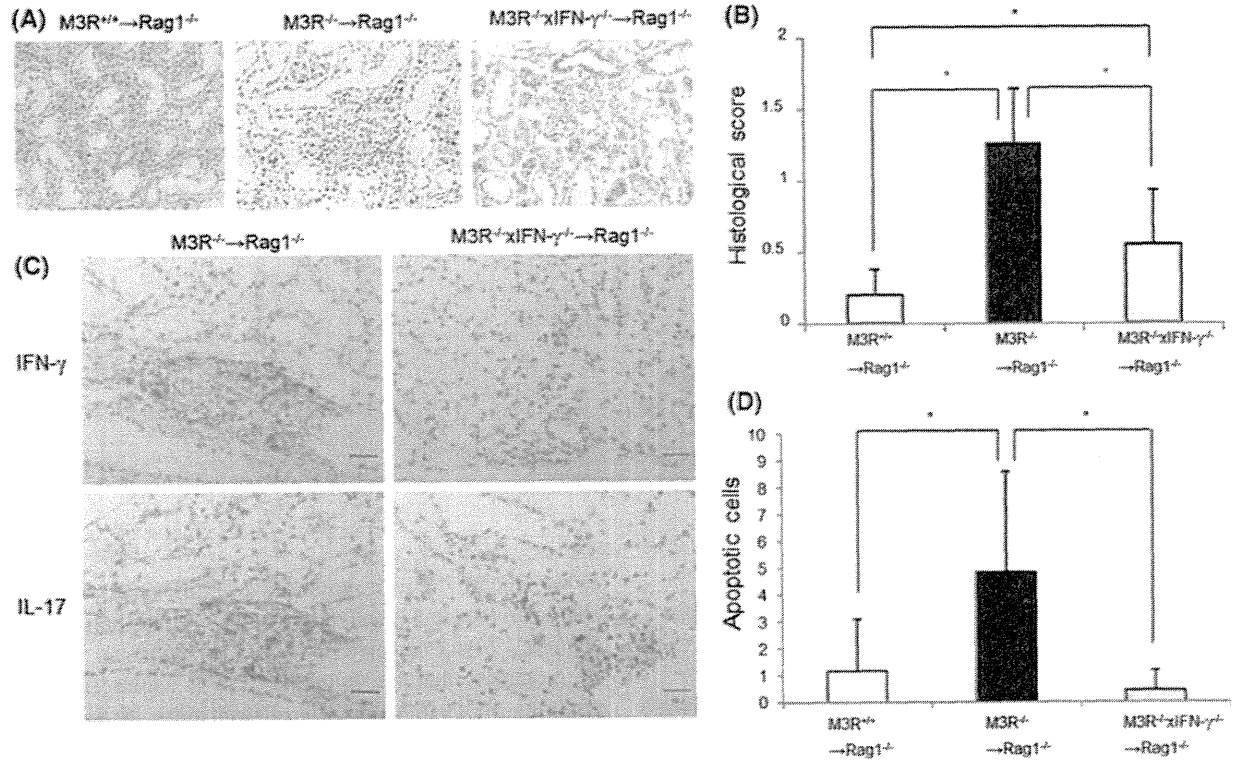

Fig. 1 MIS was reduced in $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice. **a** Salivary glands isolated from $Rag1^{-/-}$ mice at day 45 after inoculation of splenocytes from $M3R^{+/+}$, $M3R^{-/-}$, and $IFN\text{-}\gamma^{-/-}$/$M3R^{-/-}$ mice immunized with M3R peptides encoding the extracellular domains of M3R on days 0 and 10. On the day of immunization, 500 ng of pertussis toxin were injected intraperitoneally. Ten days after booster immunization, the spleens were isolated and transferred into $Rag1^{-/-}$ mice. The salivary glands from $M3R^{+/+} \rightarrow Rag1^{-/-}$, $M3R^{-/-} \rightarrow Rag1^{-/-}$, and $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice were sliced into 4-μm-thick sections, and each section was stained with Mayer's hematoxylin and eosin (H&E). The original magnification was ×100. Representative images of 3–5 mice from each group. **b** The infiltrating cells in salivary glands from $M3R^{+/+} \rightarrow Rag1^{-/-}$, $M3R^{-/-} \rightarrow Rag1^{-/-}$ and $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice were estimated by histological score. The mean + SD are shown. $^*P < 0.05$ (Student's t test). **c** Immunohistochemical analysis of IFN-γ and IL-17 in salivary glands of $M3R^{-/-} \rightarrow Rag1^{-/-}$ and $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice. The stained sections were counterstained with H&E, and were observed at 30× the original magnification. Representative images of three mice from each group. **d** Apoptotic cells in salivary glands from $M3R^{+/+} \rightarrow Rag1^{-/-}$, $M3R^{+/+} \rightarrow Rag1^{-/-}$, and $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice were measured by TUNEL staining and described as the number of cells in the objective area of 4 mm^2. Data were analyzed in three fields per section. The mean + SD are shown. $^*P < 0.05$ (Student's t test)

Several studies have shown that IFN-γ is able to stimulate various cells to express Fas, which triggers apoptosis when stimulated by FasL for its ligand [9, 10]. In our study, the number of apoptotic cells in salivary glands in $M3R^{-/-}\times IFN\text{-}\gamma^{-/-} \rightarrow Rag1^{-/-}$ mice was significantly reduced compared to the number of apoptotic cells in salivary glands in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice ($P < 0.05$), although apoptotic cells were enhanced in $M3R^{-/-} \rightarrow Rag1^{-/-}$ mice in comparison with those in $M3R^{+/+} \rightarrow Rag1^{-/-}$ mice (Fig. 1c). These findings indicate that IFN-γ plays an important role in the apoptosis of epithelial cells and mononuclear cells in salivary glands in MIS mice.

In conclusion, our observations support the notion that IFN-γ-producing M3R-reactive T cells play a crucial role in the generation of SS-like sialadenitis via the induction of apoptosis. Hence, these results suggest the possibility that IFN-γ-targeting therapy could be used to regulate autoimmune sialadenitis in patients with SS.

Acknowledgments This work was supported by Health and Labour Science Research Grants (2012) for research on intractable diseases, The Clinical Study Group for Autoimmune Diseases.

Conflict of interest None.

References

1. Naito Y, Matsumoto I, Wakamatsu E, Goto D, Sugihara T, Matsumura R, et al. Muscarinic acetylcholine receptor autoantibodies in patient with Sjögren's syndrome. Ann Rheum Dis. 2005;64:510–1.
2. Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, et al. New epitopes and function of anti-M3

muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome. Clin Exp Immunol. 2010;162(1):53–61.
3. Dawson LJ, Stanbury J, Venn N, Hasdimir B, Rogers N, Smith M. Antimuscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. Arthritis Rheum. 2006;54: 1165–73.
4. Naito Y, Matsumoto I, Wakamatsu E, Goto D, Ito S, Tsutsumi A, et al. Altered peptide ligand regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjögren's syndrome. Ann Rheum Dis. 2006;65:269–71.
5. Mitsias DI, Tzioufas AG, Veiopoulou C, Zintaras E, Tassios K, Kogopoulou O, et al. The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjogren's syndrome. Clin Exp Immunol. 2002;128(3):562–8.
6. Wakamatsu E, Matsumoto I, Yasukochi T, Naito Y, Goto D, Mamura M, et al. Overexpression of phosphorylated STAT-1 alpha in the labial salivary glands of patients with Sjögren's syndrome. Arthritis Rheum. 2006;54(11):3476–84.
7. Sakai A, Sugawara Y, Kuroishi T, Sasano T, Sugawara S. Identification of IL-18 and Th17 cells in salivary glands of patients with Sjögren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. J Immunol. 2008;181(4):2898–906.
8. Iizuka M, Wakamatsu E, Tsuboi H, Nakamura Y, Hayashi T, Matsui M, et al. Pathogenic role of immune response to M3 muscarinic acetylcholine receptor in Sjögren's syndrome-like sialoadenitis. J Autoimmun. 2010;35(4):383–9.
9. Xu X, Fu XY, Plate J, Chong A. IFN-γ induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. Cancer Res. 1998;58(13):2832–7.
10. Bulosan M, Pauley KM, Yo K, Chan E, Katz J, Peck AB, et al. Inflammatory caspases are critical for enhanced cell death in the target tissue of Sjögren's syndrome before disease onset. Immunol Cell Biol. 2009;87(1):81–90.

Mod Rheumatol (2012) 22:264–271
DOI 10.1007/s10165-011-0514-8

ORIGINAL ARTICLE

Generation and functional analysis of monoclonal antibodies against the second extracellular loop of human M3 muscarinic acetylcholine receptor

Hiroto Tsuboi · Yumi Nakamura · Mana Iizuka · Naomi Matsuo · Isao Matsumoto · Takayuki Sumida

Received: 10 June 2011 / Accepted: 5 August 2011 / Published online: 30 August 2011

Abstract The M3 muscarinic acetylcholine receptor (M3R) plays a crucial role in the activation of salivary and lachrymal glands. The M3R contains four extracellular domains (the N-terminal, and the first, second, and third extracellular loops), and we recently detected antibodies against each of these four domains in a subgroup of patients with Sjögren's syndrome (SS). Functional analysis indicated that the influence of such anti-M3R antibodies on salivary secretion might differ based on the epitopes to which they bind. To clarify the relationship between B-cell epitopes on the M3R and its function, we generated two hybridomas producing anti-M3R monoclonal antibodies against the second extracellular loop of M3R (anti-M3R^{2nd} mAbs) and analyzed their function by Ca^{2+}-influx assays, using a human salivary gland (HSG) cell line. These two anti-M3R^{2nd} mAbs suppressed Ca^{2+}-influx in the HSG cells induced by cevimeline stimulation, suggesting that autoantibodies against the second extracellular loop of M3R could be involved in salivary dysfunction in patients with SS.

H. Tsuboi and Y. Nakamura contributed equally to this work.

H. Tsuboi · Y. Nakamura · M. Iizuka · N. Matsuo · I. Matsumoto · T. Sumida (✉)
Division of Clinical Immunology, Doctoral Programs in Clinical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsumida@md.tsukuba.ac.jp

Y. Nakamura
Japan Society for the Promotion of Science, Tokyo, Japan

Keywords Autoantibodies · Ca^{2+}-influx · M3 muscarinic acetylcholine receptor · Salivary secretion · Sjögren's syndrome

Introduction

Sjögren's syndrome (SS) is an autoimmune disease that affects exocrine glands including the salivary and lachrymal glands. SS is characterized by lymphocytic infiltration into these glands, leading to dry mouth and dry eyes. A number of autoantibodies, such as anti-SS-A and SS-B antibodies, are detected in patients with SS, although none identified thus far are specific to SS or associated with the disease pathology [1].

Data from recent studies suggest that some patients with SS carry inhibitory autoantibodies directed against muscarinic acetylcholine receptors, and in particular the M3 muscarinic acetylcholine receptor (M3R) [1]. To date, five muscarinic acetylcholine receptor subtypes (M1R–M5R) have been identified, but M3R is expressed in exocrine glands and plays important roles in exocrine secretion [1]. 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, and consequently, activating apical membrane Cl^- channels to induce salivary secretion (Fig. 1) [1]. Activation of M3R also induces the trafficking of aquaporin 5 (AQP5) from the cytosol to the apical membrane where it causes rapid transport of water across the cell membrane (Fig. 1) [2].

The M3R has four extracellular domains: the N-terminal region, as well as the first, second, and third extracellular loops (Fig. 1). Among these domains, the second extracellular loop is critical for receptor activation by agonists [3], and has therefore been the focus of our interest.

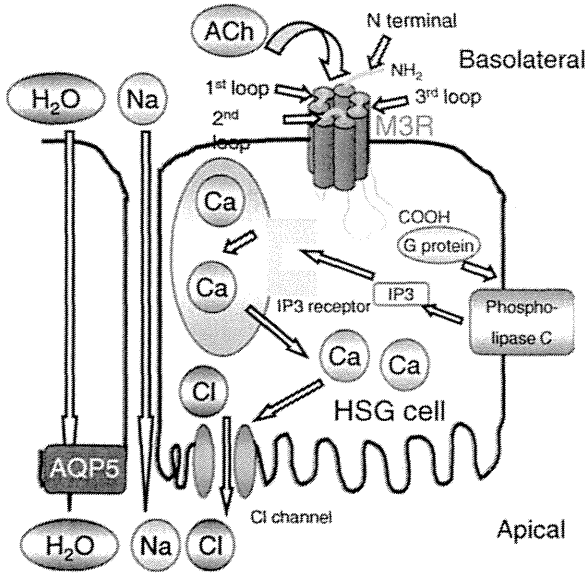

Fig. 1 The structure of M3 muscarinic acetylcholine receptor (*M3R*) and control of fluid secretion in human salivary gland (*HSG*) cells via M3R. M3R has four extracellular domains (the N-terminal region, and the first, second, and third extracellular loops), seven transmembrane domains, and intracellular domains. Acetylcholine (*ACh*) binds to and activates M3R on HSG cells, causing a rise in intracellular Ca^{2+} via inositol 1, 4, 5-trisphosphate (*IP3*) and IP3 receptors, and consequently, activating apical membrane Cl^- channels to induce salivary secretion. Activation of M3R also induces the trafficking of aquaporin 5 (*AQP5*) from the cytosol to the apical membrane where it causes rapid transport of water across the cell membrane. *N terminal* N terminal region, *1st loop* first extracellular loop, *2nd loop* second extracellular loop, *3rd loop* third extracellular loop

However, we recently showed that all four extracellular domains of the M3R were epitopes of anti-M3R antibodies in patients with SS, and that many SS patients were reactive to several extracellular domains of the M3R [4]. Moreover, we demonstrated that the influence of anti-M3R antibodies on salivary secretion might differ based on these epitopes [4]. Importantly, we showed that anti-M3R antibodies against the second extracellular loop of M3R could suppress the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) induced by agonists of the M3R, and thereby reduce salivary secretion [4].

Although our Ca^{2+}-influx assays indicated that the second extracellular loop of M3R might be the functional epitope of anti-M3R antibodies in SS patients [4], the mechanism by which such antibodies inhibit Ca^{2+}-influx, and their pathogenic roles in vivo are not clear. It is also possible that alteration of the amount or titer of anti-M3R antibodies by other antibodies in serum could influence Ca^{2+}-influx.

The present study was designed to clarify the relationship between B-cell epitopes on the M3R and its function, and to confirm that the second extracellular loop of the M3R is the functional target of anti-M3R antibodies. For this purpose, we generated two hybridomas producing anti-M3R monoclonal antibodies against the second extracellular loop of the M3R (anti-$M3R^{2nd}$ mAbs) and analyzed the function by Ca^{2+}-influx assays using a human salivary gland (HSG) cell line.

Materials and methods

Mice

Male C57BL/6j (B6) mice were purchased from Charles River Japan (Yokohama, Japan). $M3R^{-/-}$ mice, which were generated from B6 mice, were kindly provided by Dr. Minoru Matsui, MD, PhD (Tokyo-Nishi Tokushukai Hospital, Tokyo, Japan). The mice were maintained in specific pathogen-free conditions in the Laboratory of the Animal Resource Center of the University of Tsukuba. All experiments were performed according to the guide for the care and use of laboratory animals of the University of Tsukuba.

Synthesis of peptides encoding M3R extracellular domains and negative control

Peptides encoding the second extracellular loop of the human M3R ($M3R^{2nd}$, KRTVPPGECFIQFLSEPTITFG TAI), and negative control peptides (SGSGSGSGS GSGSGSGSGSGSGSGS) were synthesized chemically by a solid-phase procedure and purified by high-performance liquid chromatography (AnyGen, Gwangju, Korea). Peptides encoding other extracellular domains of the human M3R, N-terminal region ($M3R^{N1}$, MTLHNNSTTSPLF PNISSSWIHSPSDAGLP; $M3R^{N2}$, IHSPSDAGLPPGTVT HFGSYNVSRAAGNFS; $M3R^{N3}$, NVSRAAGNFSSPD GTTDDPLGGHTVWQV), the first extracellular loop of M3R ($M3R^{1st}$, FTTYIIMNRWALGNLACDLW), and the third extracellular loop ($M3R^{3rd}$, VLVNTFCDSCIPKT FWNLGY) were also synthesized (AnyGen) for peptide-blocking assays.

Immunization with M3R peptides and control peptides

B6 and $M3R^{-/-}$ mice were immunized at the base of their tails with 50 μg of $M3R^{2nd}$ peptides or control peptides emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 250 μg H37RA *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA) three times, at 2-week intervals. Anti-$M3R^{2nd}$ antibodies in the sera of these immunized mice were measured by enzyme-linked immunosorbent assay (ELISA) every 2 weeks.

Enzyme-linked immunosorbent assay for anti-M3R^{2nd} antibodies

Peptide corresponding to the sequence of M3R^{2nd} solution (100 μl/well, at 10 μg/ml) in 0.1 M Na_2CO_3 buffer, pH 9.6, was adsorbed onto Nunc-Immuno plates (Nalge Nunc International, Rochester, NY, USA) overnight at 4°C, and blocked with 5% bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline (PBS) for 1 h at 37°C. Sera from immunized mice at 1:100–1:1000 dilution in blocking buffer, or culture supernatant of hybridomas, were incubated for 2 h at 37°C. The plates were then washed twice with 0.05% Tween 20 in PBS, and 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgG (Fc; American Qualex, San Clemente, CA, USA) diluted 1:1000 in PBS was added for 1 h at room temperature. After three washes, 100 μl of *p*-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) solution (final concentration 1 mg/ml) was added as the alkaline phosphate substrate. The plates were incubated for 30 min at room temperature in the dark, and the optical density at 405 nm was measured by plate spectrophotometry. Optical density was used to express the titer of anti-M3R^{2nd} antibodies. Measurements were performed in triplicate and standardized between experiments by using positive control serum.

Generation of anti-M3R^{2nd} monoclonal antibodies

We generated monoclonal antibodies (mAbs) against human M3R^{2nd} according to the A Shibuya Lab method (Department of Immunology, Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan). In brief, 6 weeks after the first immunization, splenocytes were harvested from immunized mice and fused with an SP2/0 myeloma cell line by a polyethyleneglycol (PEG) method. Anti-human M3R^{2nd} mAbs-positive hybridoma clones were screened by the ELISA assay described above. The hybridomas were cultured in medium E or Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1000 U/ml of penicillin, and 100 μg/ml of streptomycin. The anti-M3R^{2nd} mAbs were purified from the culture supernatant of positive hybridoma clones using a Protein G column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and the purity was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The mAb isotype was determined using an IsoStrip kit (Roche Diagnostics, Roche Applied Science, Penzberg, Germany).

RNA extraction and complementary DNA synthesis

Total RNA was extracted from 1×10^7 anti-M3R^{2nd} mAb-producing hybridoma cells by the ISOGEN method (Nippon Gene, Tokyo, Japan). The complementary DNA (cDNA) was synthesized using a Revert Aid™ First Standard cDNA Synthesis kit (Fermentas, Burlington, ON, Canada).

Reverse transcription polymerase chain reaction (RT-PCR)

The primer sequences used were as follows: mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-AACTTTGGCATTGTGGAAGG-3′ (forward), 5′-ACACATTGGGGGTAGGAACA-3′ (reverse); Cγ, 5′-TCTAGAATTCGGCCAGTGGATAGA-3′; V_H558 family, 5′-TCTAGAATTCGCAGGTGTCCACTCC-3′ or 5′-TCTAGAATTCTCCGAGGTTCAGCTG-3′; V_H7183 family, 5′-TCTAGAATTCGCTGGTGGAGTCTGG-3′; V_H36–60 family, 5′-TCTAGAATTCGGGTATCCTGTCAGA-3′; C_κ, 5′-TCTAGAATTCTGCAGCATCAGCCC-3′; V_κ5 group, 5′-TCTAGAATTCATAATGACCAGAGGA-3′; V_κ8 group, 5′-TCTAGAATTCATTGTGATGACACAG-3′; V_κ12 and 13 group, 5′-TCTAGAATTCGCCAGATGTGACATC-3′;V_κ22 group, 5′-TCTAGAATTCGGAGACATTGTGATG-3′; V_κ23 group, 5′-TCTAGAATTCAGCCTCCAGAGGTGA-3′. The PCR for GAPDH was performed for 30 cycles at 94°C for 30 s to denature, 60°C for 30 s to anneal, 72°C for 30 s for extension, and a final 7-min extension at 72°C. The PCR for V_H and V_κ family genes was performed for 40 cycles at 94°C for 1.5 min to denature, 60°C for 1 min to anneal, 72°C for 1 min for extension, and a final 7-min extension at 72°C. PCR products were separated on 2% agarose gels, and amplified DNA bands encoding $V_HDJ_HC_H$ or $V_\kappa J_\kappa C_\kappa$ genes were identified.

Cloning and sequencing of cDNA encoding the V_H and V_κ genes

The PCR products were excised from the agarose gel, and DNA bands in the range of 400 bp were purified using a Mini Elute gel extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were cloned into the TOPO-TA cloning vector (Invitrogen, San Diego, CA, USA), and randomly picked clones were screened for the insert. Positive clones were used for cycle sequencing, using a Big Dye terminator cycle sequence kit (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the manufacturer. The sequences were determined by capillary sequencing (Applied Biosystems 310 genetic analyzer). The Ig BLAST system (National Institutes of

Health) and the international ImMunoGeneTics information system (IMGT) were used to identify the Ig-VDJ germline sequence.

Western blot analysis

We generated human M3R transfected *Escherichia coli*, and we purified M3R protein conjugated with His-tag from the cell lysate of M3R protein-producing *E. coli* by affinity chromatography. Human recombinant M3R proteins (hr-M3R) were fractionated on SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 100% Block-Ace (Dainippon, Osaka, Japan) for 1 h, and then incubated with mouse anti-human $M3R^{2nd}$ mAbs or mouse IgG2bκ mAbs (isotype control). Horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody (1:2000 dilution; Dako, Tokyo, Japan) was applied as the secondary antibody for 30 min. The proteins were detected by enhanced chemiluminescence using an ECL Western blot detection kit (Amersham, currently a subsidiary of GE Healthcare, Buckinghamshire, UK).

M3R peptide-blocking ELISA for anti-$M3R^{2nd}$ antibodies

Anti-$M3R^{2nd}$ mAbs were pre-absorbed with 1–100 μg/ml of peptides encoding M3R extracellular domains (N-terminal, first, second, and third extracellular loop) for 2 h at 37°C, and were used in subsequent ELISA assays as the primary antibodies.

Ca^{2+}-influx assay for anti-$M3R^{2nd}$ mAbs

An HSG cell line, which was generated from a human salivary tumor, was kindly provided by Professor Ichiro Saito (Tsurumi University). HSG cells (15,000–20,000 cells/well) were precultured in 96-well plates for fluorescence assays for 48 h at 37°C. The cells were then pre-incubated with purified anti-$M3R^{2nd}$ mAbs or isotype control for 12 h. Antibodies were washed off, and the HSG cells were loaded with Fluo-3, a fluorescence probe for calcium, for 1 h. After further washing, the HSG cells were analyzed for Ca^{2+}-influx. This involved stimulating the HSG cells with cevimeline hydrochloride, an M3R-specific agonist, at a final concentration of 20 mM. Changes in

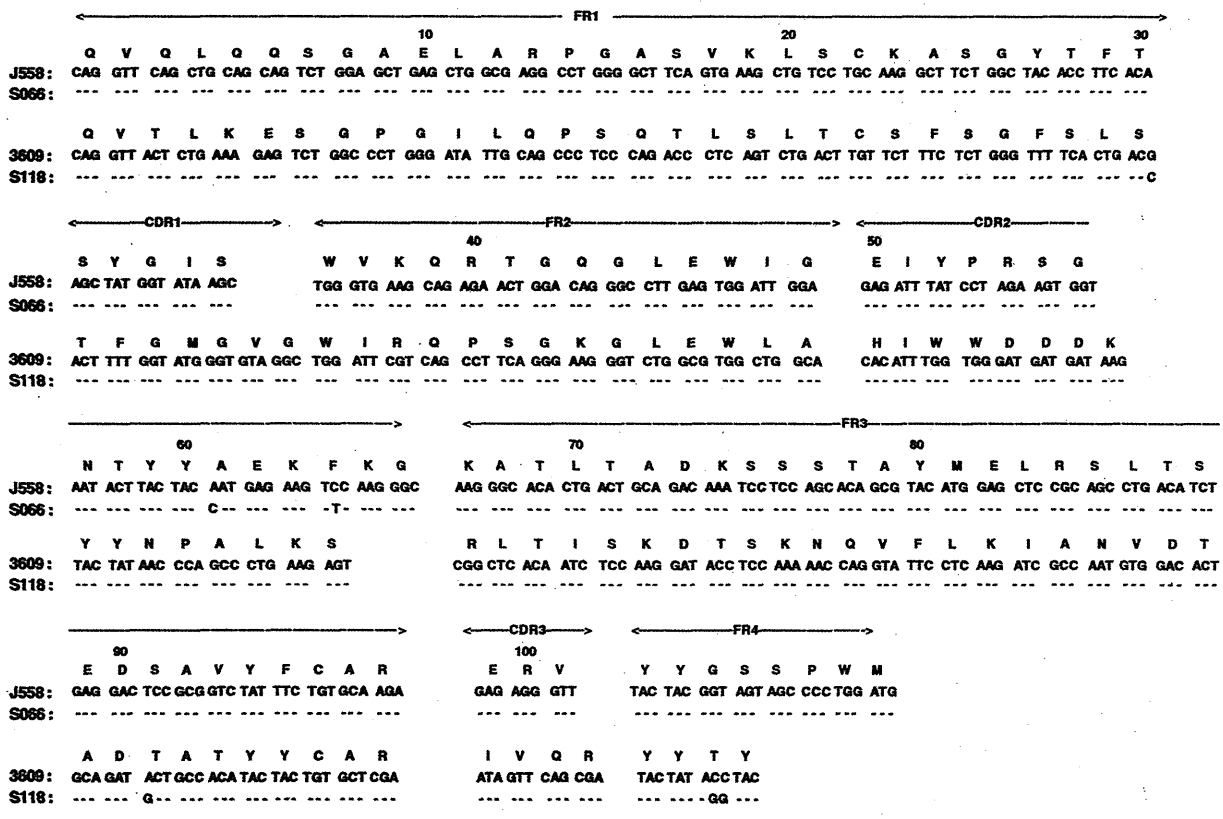

Fig. 2 Sequences of V_H regions of hybridoma clones producing anti-$M3R^{2nd}$ monoclonal antibodies (mAbs). V_H gene sequences of S066 and S118 hybridoma clones producing anti-$M3R^{2nd}$ mAbs were compared with J558 V_H gene and 3609 V_H gene germline sequences

$[Ca^{2+}]i$ in HSG cells were measured on a fluorescence plate reader.

Statistical analysis

Data were expressed as means ± SD. Differences between groups were examined for statistical significance using Mann–Whitney's *U*-test. *P* values of less than 0.05 were considered statistically significant.

Results

Generation of anti-M3R^{2nd} monoclonal antibodies

The titers of anti-M3R^{2nd} antibodies were increased in mice immunized with M3R^{2nd} peptides, but not in mice immunized with control peptides (data not shown). Splenocytes from mice showing a high titer of anti-M3R^{2nd} antibodies were fused with myeloma cells to generate the two positive hybridoma clones (S066 and S118) producing mAbs against human M3R^{2nd}. Both were IgG2bκ isotype (data not shown).

Sequencing of the V_H and V_κ regions of hybridoma clones producing anti-M3R^{2nd} mAbs

Sequencing of the V_H and V_κ regions of the two positive hybridoma clones showed that the S066 clone used the J558 V_H and 19 V_κ genes, while the S118 clone used the 3609 V_H and 21 V_κ genes (Figs. 2, 3). The V_H region of clone S066 had two point mutations in the CDR2 domain by comparison with the J558 V_H gene germline sequence (Fig. 2), while the V_H region of clone S118 had four point mutations in the FR1, FR3, and FR4 domains by comparison with the 3609 V_H gene germline sequence (Fig. 2). The V_κ region of clone S066 had one point mutation in the FR2 domain by comparison with the 19 V_κ gene germline sequence (Fig. 3), while the V_κ region of clone S118 had seven point mutations by comparison with the 21 V_κ gene germline sequence (Fig. 3). This sequence analysis showed that our two hybridoma clones (S066 and S118) had different V_H and V_κ regions including the CDR3 domains.

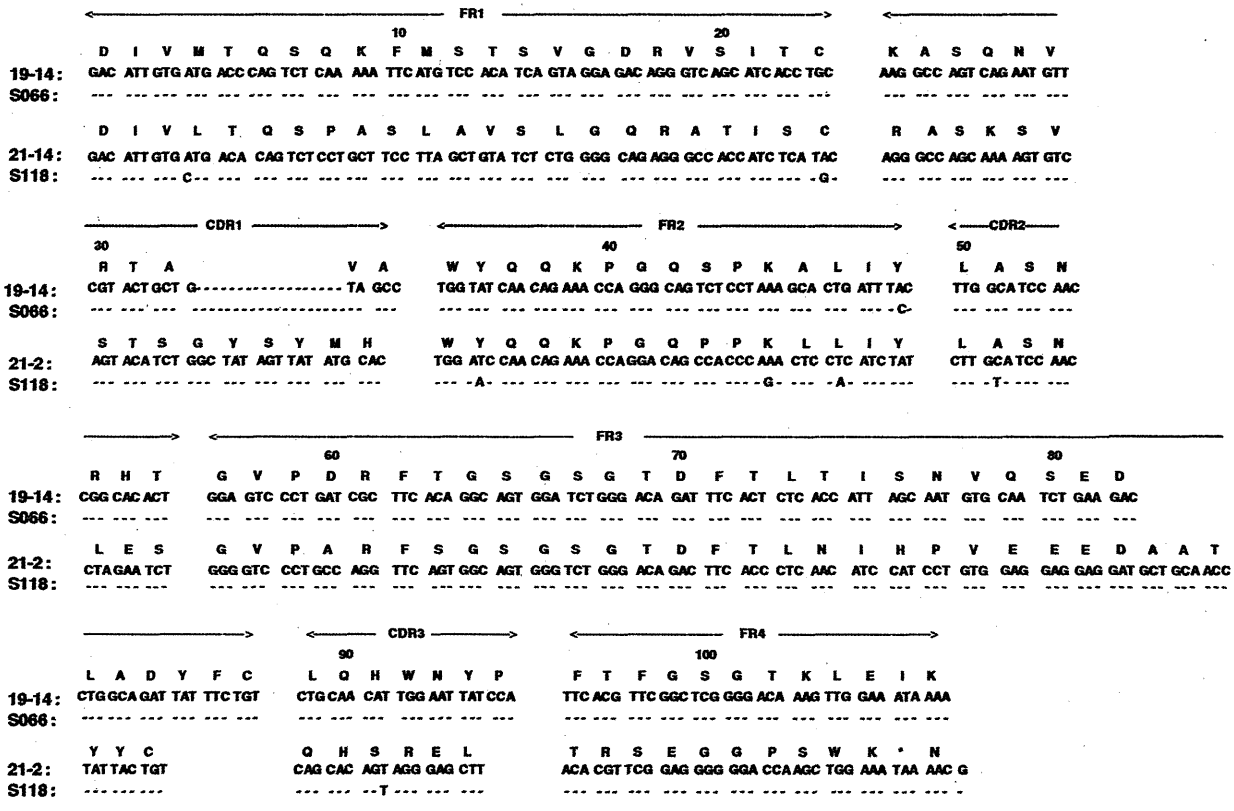

Fig. 3 Sequences of V_κ regions of hybridoma clones producing anti-M3R^{2nd} mAbs. V_κ gene sequences of S066 and S118 hybridoma clones were compared with 19 V_κ gene and 21 V_κ gene germline sequences

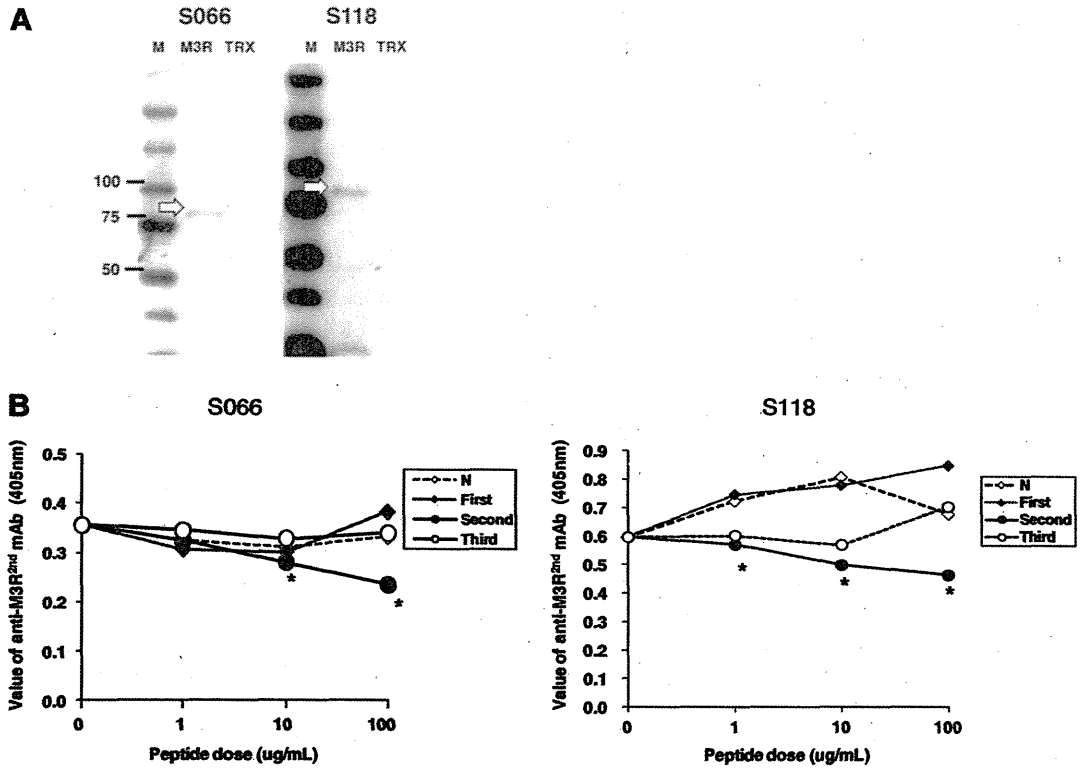

Fig. 4 **a** Binding of anti-M3R^{2nd} mAbs to human recombinant M3R proteins (hr-M3R). Western blot analysis showing the binding of anti-M3R^{2nd} mAbs produced by S066 and S118 hybridoma clones to hr-M3R, but not to TRX control protein. **b** M3R peptide-blocking enzyme-linked immunosorbent assay (ELISA) for anti-M3R^{2nd} antibodies. Pre-incubation with M3R^{2nd} peptide, but not with other M3R peptides, inhibited the detection of anti-M3R^{2nd} mAbs by ELISA in a peptide-dose-dependent manner. $^{*}P < 0.05$ versus without peptide pre-incubation (Mann–Whitney's *U*-test)

Binding of anti-M3R^{2nd} mAbs to human recombinant M3R protein

Western blot analysis proved that anti-M3R^{2nd} mAbs produced by hybridoma clones S066 and S118 bound to hr-M3R, but not to TRX control protein (Fig. 4a).

Specificity of anti-M3R^{2nd} mAbs for M3R^{2nd}

The M3R peptide-blocking ELISA for anti-M3R^{2nd} antibodies showed that pre-incubation with the M3R^{2nd} peptide, but not with other M3R peptides, inhibited the detection of anti-M3R^{2nd} mAbs by ELISA in a peptide dose-dependent manner (Fig. 4b).

Inhibition of Ca^{2+}-influx in HSG cells by anti-M3R^{2nd} mAbs

Figure 5a shows that Ca^{2+}-influx was enhanced in a cevimeline dose-dependent manner without pre-incubation of anti-M3R^{2nd} mAbs or isotype control (Fig. 5a). The anti-M3R^{2nd} mAbs inhibited the Ca^{2+}-influx in HSG cells induced by cevimeline stimulation by 21% (clone S066) and 16% (clone S118) (Fig. 5b). The rate of inhibition was significantly higher for anti-M3R^{2nd} mAbs than for the isotype control ($P < 0.05$, Mann–Whitney's *U*-test) (Fig. 5b).

Discussion

Anti-M3R antibodies have become a recent focus of interest in rheumatology, because of their potential roles in pathogenesis and diagnosis, and as therapeutic targets in patients with SS [1]. Herein we report the generation of hybridoma clones (S066 and S118) that produced anti-M3R^{2nd} mAbs. Sequence analysis indicated that the two hybridoma clones were different, based on different V_H and V_κ regions including the CDR3 domains. We also showed, in western blot analysis, that both anti-M3R^{2nd} mAbs specifically bound to hr-M3R protein. Moreover, the detection of anti-M3R^{2nd} mAbs by ELISA was inhibited by

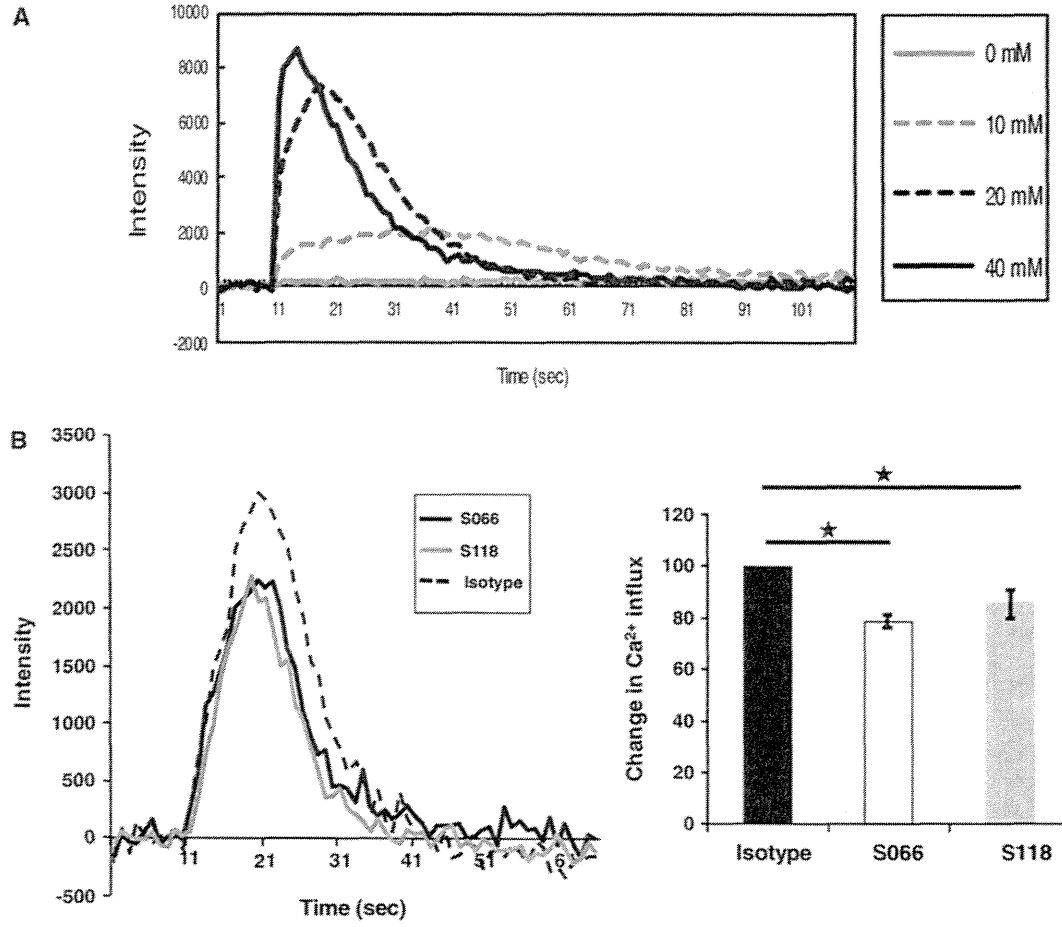

Fig. 5 Ca^{2+}-influx assay for anti-$M3R^{2nd}$ mAbs. **a** The traces show changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) after stimulation with cevimeline, without pre-incubation of anti-M3R mAbs or isotype control. Change in $[Ca^{2+}]i$ was enhanced in a cevimeline-dose-dependent manner. We selected a final concentration of 20 mM for the Ca^{2+}-influx assay. *0*, *10*, *20*, *40* mM; final concentrations of cevimeline. **b** The traces show changes in $[Ca^{2+}]i$ after stimulation with cevimeline. Representative data in S066, S118, and isotype control. The histogram shows the mean ± SD values of maximum change in $[Ca^{2+}]i$ (peak $[Ca^{2+}]i$ − baseline $[Ca^{2+}]i$) induced by cevimeline in S066, S118, and isotype control, shown as a ratio compared to maximum change in $[Ca^{2+}]i$ in isotype control. The maximum change in isotype control is described as 100. Data are averages of triplicates from three independent experiments. $^*P < 0.05$ versus isotype control (Mann–Whitney's *U*-test)

the $M3R^{2nd}$ peptide, verifying the specificity of our mAbs to $M3R^{2nd}$ among the extracellular domains of M3R. Importantly, both anti-$M3R^{2nd}$ mAbs also inhibited the Ca^{2+}-influx in HSG cells induced by cevimeline stimulation. Together, these data suggest that our anti-$M3R^{2nd}$ mAbs specifically recognize both the conformational and linear forms of the $M3R^{2nd}$ epitope, and can inhibit cellular Ca^{2+}-influx.

The anti-$M3R^{2nd}$ mAbs described here could prove to be valuable and important in analyzing the pathogenic roles of anti-M3R autoantibodies in SS. First, using these antibodies in functional assays could clarify the relationship between epitopes and function more distinctly than using IgG from M3R autoantibody-positive SS patients. Indeed, using IgG derived from sera makes it difficult to exclude that other antibodies in serum influence the functional assay. Several methods have been used to functionally analyze anti-M3R antibodies. For example, assays of smooth muscle showed that IgG fractions from patients with SS (SS-IgG) inhibited carbachol-evoked or nerve-evoked bladder or colon contractions, respectively [5, 6]. In salivary gland cells, SS-IgG inhibited both the rise in $[Ca^{2+}]i$ induced by carbachol and the pilocarpine-induced AQP5 trafficking to the apical membrane from the cytoplasm [2]. The inhibitory actions were also acutely reversible [7]. In addition, Koo et al. [8] have reported that, in M3R peptide-blocking Ca^{2+}-influx assays using HSG cells, the third extracellular loop of M3R is a functional epitope bound by SS-IgG. In contrast, in the present study, the anti-$M3R^{2nd}$ mAbs suppressed Ca^{2+}-influx, indicating

that the second extracellular loop of M3R might also be a functional epitope. Interestingly, this result accords with our previous observations showing that IgG from anti-M3R^{2nd} antibody-positive SS patients inhibited Ca^{2+}-influx in HSG cells [4]. We selected cevimeline hydrochloride as the M3R agonist, and not carbachol which was used in other studies [2, 8], because cevimeline hydrochloride had higher selectivity for M3R than other agonists such as carbachol. Final clarification of the relationship between the epitopes of M3R and its function requires that mAbs are also generated against the other M3R extracellular domains (N-terminal, and first and third extracellular loops).

Second, anti-M3R^{2nd} mAbs could contribute to our understanding of the molecular mechanism underlying Ca^{2+}-influx, and further experiments on the effects of anti-M3R^{2nd} mAbs on M3R signaling, binding to M3R agonists, and the influence on M3R expression are needed. Third, using our anti-M3R^{2nd} mAbs experimentally could clarify the pathogenic roles of anti-M3R antibodies in vivo. Robinson et al. [9] reported that the transfer of SS-IgG to mice reduced saliva production, via SS-IgG competitively inhibiting the binding of muscarinic receptor agonist to salivary gland membranes. Because the amino acid sequence of mouse M3R^{2nd} completely accords with that of human M3R^{2nd}, our anti-human M3R^{2nd} mAbs could recognize mouse M3R molecules. To clarify the pathogenic roles of anti-M3R antibodies in vivo, we need to transfer our anti-human M3R^{2nd} mAbs to immunodeficient mice, and then investigate secretory function and general pathology in the salivary glands.

In conclusion, we generated two hybridomas producing anti-M3R^{2nd} mAbs that suppressed the Ca^{2+}-influx in HSG cells induced by cevimeline stimulation. These results suggest that anti-M3R autoantibodies against the second extracellular loop of M3R could play a role in the salivary dysfunction of patients with SS.

Conflict of interest None.

References

1. Dawson L, Tobin A, Smith P, Gorton T. Antimuscarinic antibodies in Sjögren's syndrome. Arthritis Rheum. 2005;52:2984–95.
2. Li J, Ha YM, Ku NY, Choi SY, Lee SJ, Oh SB, et al. Inhibitory effects of autoantibodies on the muscarinic receptors in Sjögren's syndrome. Lab Invest. 2004;84:1430–8.
3. Scarselli M, Li B, Kim SK, Wess J. Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. J Biol Chem. 2007;282:7385–96.
4. Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, et al. New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome. Clin Exp Immunol. 2010;161:53–61.
5. Waterman SA, Gordon TP, Rischmueller M. Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. Arthritis Rheum. 2000;43:1647–54.
6. Cavill D, Waterman SA, Gordon TP. Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjögren's syndrome. Scand J Immunol. 2004;59:261–6.
7. Dawson LJ, Stanbury J, Venn N, Hasdimir B, Rogers SN, Smith PM. Antimuscarinic antibodies in primary Sjögren's syndrome reversibly inhibit the mechanism of fluid secretion by human submandibular salivary acinar cells. Arthritis Rheum. 2006;54:1165–73.
8. Koo NY, Li J, Hwang SM, Choi SY, Lee SJ, Oh SB, et al. Functional epitopes of muscarinic type3 receptor which interacts with autoantibodies from Sjögren's syndrome patients. Rheumatology. 2008;47:828–33.
9. Robinson CP, Brayer J, Yamachika S, Esch TR, Peck AB, Stewart CA, et al. Transfer of human serum IgG to nonobese diabetic Igμ^{null} mice reveals a role for autoantibodies in the loss of secretory function of exocrine tissues in Sjögren's syndrome. Proc Natl Acad Sci USA. 1998;95:7538–43.

Tsuboi et al. Arthritis Research & Therapy 2012, 14:R171
http://arthritis-research.com/content/14/4/R171

arthritis research&therapy

RESEARCH ARTICLE Open Access

Analysis of IgG4 class switch-related molecules in IgG4-related disease

Hiroto Tsuboi[1], Naomi Matsuo[1], Mana Iizuka[1], Sayaka Tsuzuki[1], Yuya Kondo[1], Akihiko Tanaka[2], Masafumi Moriyama[2], Isao Matsumoto[1], Seiji Nakamura[2] and Takayuki Sumida[1*]

Abstract

Introduction: Immunoglobulin G4 (IgG4)-related disease (IgG4-RD) is a new disease entity characterized by high serum IgG4 levels, IgG4-positive plasmacytic infiltration, and fibrosis in various organs. The purpose of this study was to determine the mechanism of upregulation of IgG4 class switch recombination in IgG4-RD.

Methods: We extracted RNA from peripheral blood mononuclear cells (PBMCs) of patients with IgG4-RD ($n = 6$), Sjögren syndrome (SS) ($n = 6$), and healthy controls ($n = 8$), from CD3-positive T cells and CD20-positive B cells sorted from PBMCs of patients with IgG4-RD ($n = 3$), SS ($n = 4$), and healthy controls ($n = 4$), as well as from labial salivary glands (LSGs) of patients with IgG4-RD ($n = 11$), SS ($n = 13$), and healthy controls ($n = 3$). The mRNA expression levels of IgG4-specific class switch-related molecules, such as Th2 cytokines (IL-4 and IL-13), Treg cytokines (IL-10 and TGF-β), and transcriptional factors (GATA3 and Foxp3) were examined with quantitative polymerase chain reaction (PCR). IgG4-nonspecific class switch-related molecules, such as CD40, CD154, BAFF, APRIL, IRF4, and AID, were also examined.

Results: The expression levels of Treg cytokines (IL-10 and TGF-β) and AID were significantly higher in LSGs of IgG4-RD than in SS and the controls ($P < 0.05$, each). In contrast, those of CD40 and CD154 were significantly lower in PBMCs of IgG4-RD than in SS ($P < 0.05$, each), whereas CD40 in CD20-positive B cells and CD154 in CD3-positive T cells were comparable in the three groups.

Conclusion: Overexpression of IL-10, TGF-β, and AID in LSGs might play important roles in the pathogenesis of IgG4-RD, such as IgG4-specific class-switch recombination and fibrosis. IgG4 class-switch recombination seems to be mainly upregulated in affected organs.

Introduction

IgG4-related disease (IgG4-RD) is a new disease entity characterized by high serum IgG4 levels, infiltration of IgG4-positive plasmacytes, and fibrosis of various organs, such as pancreas, bile duct, salivary and lacrimal glands, thyroid, lung, liver, kidney, prostate, aorta, retroperitoneum, and lymph nodes [1,2]. Although the clinical features including serum abnormalities, organ involvement, diagnosis, and the therapeutic approach have been reported recently [1,2], the pathogenesis of this disease, including the roles of high IgG4 and IgG4-positive plasmacytes, and the molecular mechanism involved in the upregulation of IgG4 class-switch recombination remains unclear.

Recent studies reported increased proportions of type 2 helper T (Th2) cells and regulatory T (Treg) cells and increased production levels of Th2 and Treg cytokines in tissues of IgG4-RD, such as sclerosing pancreatitis and cholangitis [3], sialadenitis [4,5], and tubulointerstitial nephritis [6]. Moreover, high Th2 cell count and overproduction of Th2 cytokines have been described in peripheral blood of IgG4-RD [4,7], as well as in Treg cells [8].

It is reported that Th2 cytokines (IL-4 and IL-13) and Treg cytokine (IL-10) can induce IgG4- and IgE-specific class-switch recombination [9,10], and tumor growth factor (TGF)-β, a Treg cytokine, could induce tissue fibrosis [11]. Thus, increased production of IL-4, IL-13, IL-10, and TGF-β could contribute to the pathogenesis of IgG4-RD,

* Correspondence: tsumida@md.tsukuba.ac.jp
[1]Department of Internal Medicine, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, 305-8575, Japan
Full list of author information is available at the end of the article