

A Potential Role of Autoantibodies against Muscarinic Type 3 Receptor in Pathogenesis of Sjögren's Syndrome

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Abstract

Sjögren's Syndrome (SS) is a systemic autoimmune disease characterized by focal mononuclear cell infiltration into target organs such as salivary and lacrimal glands, leading to sicca symptoms. SS is characterized by the production of numerous circulating autoantibodies. The anti-muscarinic autoantibodies against the type 3 receptor (anti-M3R autoantibodies) in primary SS patients' sera has recently been studied as its possible involvement in the pathogenesis of SS and a capable diagnostic marker, since M3R plays critical roles in exocrine secretion and smooth muscle contraction. Anti-M3R autoantibodies in the sera of SS patients were detectable with high sensitivity and specificity and had broad inhibitory effects on the exocrine secretion and the smooth muscle contraction mediated by parasympathetic neurons. In Salivary Gland Epithelial Cells (SGECs), the inhibitory effect of SS autoantibodies on M3R function were verified at the cellular and molecular levels; anti-M3R autoantibodies inhibited carbachol-induced $[Ca^{2+}]_i$ increase and the activities of ion channels and membrane transporters, that are dependent on $[Ca^{2+}]_i$. Anti-M3R autoantibodies appear to inhibit M3R function in two ways: acute desensitization of M3R by direct occupation of agonist binding sites of the receptor and the resulting receptor internalization. Abundant anti-M3R autoantibodies may bind to the remaining unoccupied M3Rs, thereby causing progressive and long-term loss of M3R function. Anti-M3R positivity in SS patients was also associated with some clinical manifestations such as leucopenia, anemia, and thrombocytopenia. In this review, we will discuss the prevalence of anti-M3R autoantibodies in the sera of SS patients, the inhibitory effect of anti-M3R autoantibodies in various tissues, and the potential role of anti-M3R autoantibodies with SGECs in the pathogenesis of SS.

Keywords: Sjögren's syndrome; Muscarinic type 3 receptor; Autoantibodies

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration of the salivary and lacrimal glands, leading to xerostomia and xerophthalmia. As a systemic disease, SS also has diverse extraglandular organ involvement including in the gastrointestinal tract, lung, renal, bladder, blood vessels, and peripheral and central nervous system. SS can be divided into primary SS (pSS) and secondary SS, in which the sicca symptoms are associated with other autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, or systemic sclerosis [1,2]. Patients with SS have inflammatory lesions in their target organs characterized by lymphocytic infiltration at the focal, periductal and perivenular sites with abnormal T and B cell activity. The majority of the infiltrating lymphocytes are T and B cells and less commonly macrophages, dendritic cells and NK-cells. Generally, the proportion of the infiltrating mononuclear cells is associated with the severity of the autoimmune lesion [3].

The prevalence of various autoantibodies in the sera of patients with SS is one of the hallmarks of this disease [4]. Others include the presence of anti-Ro and anti-La antibodies, which are included in the classification criteria for primary SS [5]. Anti-nuclear autoantibodies

(ANA) and rheumatoid factor (RF) are also present with high prevalence in primary SS [6]. In addition, other autoantibodies found in SS patients are cryoglobulins, anti-smooth muscle antibodies (ASMA), anti-mitochondrial antibodies (AMA) [7,8], anti-centromere antibodies (ACA) [9], antibodies against cyclic citrullinated peptides (anti-CCP) [10], anti-carbonic anhydrase antibodies [11], anti- α fodrin antibodies [12] and anti-muscarinic autoantibodies [13-17]. These autoantibodies can be diagnostic or prognostic markers and are associated with some specific extra-glandular manifestations. The saliva and tears of patients with primary SS also contain high levels of autoantibodies such as anti-Ro/SSA, anti-La/SSB and anti- α fodrin antibodies [18,19].

The presence of autoantibodies against muscarinic type 3 receptors (anti-M3R autoantibodies) in the sera of patients with SS has been investigated by several researchers in the last two decades and is an emerging candidate to be part of the diagnostic criteria for primary SS due to its high specificity [20,21]. However, the sensitivity of anti-M3R autoantibodies is extremely variable ranging from 0% to 97% depending on the methods used to detect them (Table 1). To date, anti-M3R autoantibodies are also considered to have a role in the pathogenic mechanism of pSS [22]. In this review, we will focus on anti-M3R autoantibodies in detail to discuss the prevalence, function, and further potential pathogenic roles of anti-M3R autoantibodies in SS.

Patients group	Peptide sequence	Method	Sensitivity (%)	Year	Reference
pSS	213-237	ELISA	0	2002	[23]
pSS	213-228	ELISA	77.5	2005	[24]
	213-228 (GST)		97		
pSS	213-237	ELISA	9	2005	[25]
sSS			14		
pSS	213-228 (GST)	ELISA	90	2005	[21]
sSS			29		
pSS	514-527	BIA-core	NA	2008	[15]
	228-237				
SS	1-68	ELISA	42.9	2010	[27]
	125-144		47.6		
	213-237		54.8		
	511-530		45.2		
pSS	205-220 (cyclic)	ELISA	62.2	2011	[26]
	205-220 (linear)		56.1		
pSS	184-227 (GST)	ELISA	75.6	2013	[20]
	506-521 (GST)		86.5		
pSS	205-221 (cyclic)	ELISA	50	2014	unpublished
	520-527 (cyclic)		58.3		

pSS: primary SS; sSS: secondary SS; NA: Not Available

Table 1: Peptide-based detection of anti-M3R autoantibodies in SS patients.

The Prevalence of Anti-M3R Autoantibodies in Patients with pSS

The presence of circulating anti-M3R autoantibodies with muscarinic cholinergic activity in pSS has been reported by Bacman et al. [17]. Using a ³H-QNB binding assay, they found that IgG from patients with pSS (pSS IgG) specifically bind to rat parotid gland membranes in a way similar to that of carbachol, a cholinergic agonist. They further confirmed that this binding disrupts cholinergic signal transduction. Thus, the first finding regarding the presence of anti-M3R autoantibodies in SS was achieved by functional approaches. Since then, the prevalence of anti-M3R autoantibodies in patients with SS has been investigated by several researchers in different ways mainly using ELISA. Although one study failed to detect anti-M3R autoantibodies with M3R peptide-biotin and streptavidin coated ELISA [23], anti-M3R autoantibodies against the peptide of M3R²¹³⁻²²⁸, corresponding to the second extracellular loop of M3R, was successfully detected in the sera of patients with pSS using a synthetic dimerized peptide or GST-peptide fusion protein with ELISA [24]. The recombinant fusion protein showed 97% sensitivity in detecting anti-M3R autoantibodies in pSS patients. However, the variation in the sensitivity was quite large, ranging from 0% to 97% (Table 1). Naito et al. [25] observed only 11/122 (9%) anti-M3R

positivity in pSS patients using the M3R²¹³⁻²³⁷ peptide, while Kovács et al. [21] found that 66 out of 73 (90%) pSS patients were anti-M3R²¹³⁻²²⁸-positive, using a peptide-GST fusion protein. He et al. [26] demonstrated that the sensitivity (62.2%) and specificity (95.1%) of the cyclic M3R²⁰⁵⁻²²⁰ peptide were higher than that of the linear peptide (56.1% and 84.7%, respectively). These results indicate that the physiologic structure of M3R peptides such as in the case of GST-fusion protein or dimerized peptide or cyclic peptides is important to detect the exact epitopes with high sensitivities. The specificity of anti-M3R autoantibodies in SS was also variable but in a high range from 58.1% to 100%, compared to healthy controls [20,21,26]. The functional epitopes on M3R to the anti-M3R autoantibodies in patients with pSS are also variable depending on the method used. Koo et al. [15] found a new epitope on the third extracellular loop of M3R⁵¹⁴⁻⁵²⁷ that specifically interacted with pSS autoantibodies using a BIAcore system. We further confirmed that both cyclized peptides, which correspond to parts of the second (M3R²⁰⁵⁻²²¹) and third (M3R⁵²⁰⁻⁵²⁷) extracellular loop of M3R, showed high reactivity with pSS IgG using ELISA (unpublished data). Notably, Deak et al. [20] reported that a GST-YNIP (M3R⁵⁰⁶⁻⁵²¹) fusion peptide showed higher sensitivity (86.5%) and specificity (100%) than those of GST-AGSE (M3R¹⁸⁴⁻²²⁷) (75.6% and 58.1%, respectively) in pSS patients. Tsuboi et al. [27] found that variable autoantibodies were detectable against the

N-terminal (M3R¹⁻⁶⁸), first (M3R¹²⁵⁻¹⁴⁴), second (M3R²¹³⁻²³⁷) and third (M3R⁵¹¹⁻⁵³⁰) loops of M3R with sensitivities of 42.9%, 47.6%, 54.8% and 45.2%, respectively, in SS patients.

Inhibition of Muscarinic Receptor Function in Various Tissues by SS Autoantibodies

Although detection of anti-M3R autoantibodies in patients with SS using immunoreactive methods is controversial, various functional approaches have revealed the presence of anti-M3R autoantibodies in SS. The functional assays were mainly performed using contractile smooth muscle cells or salivary gland epithelial cells (SGEC), in which M3R plays a key role in muscle contraction or salivary secretion. Patients' sera with pSS had an inhibitory effect on the M3R-induced contraction of bladder smooth muscle strips and the same sera even had acute agonist effects before its inhibitory effects [28]. Furthermore, anti-muscarinic autoantibodies in IgG fractions have an inhibitory effect on nerve-evoked bladder contractions and that 8 out of 11 SS patients with anti-muscarinic autoantibodies manifested bladder irritability. As in an aforementioned report [27], researchers combined ELISA and functional assay methods to show that having an excitatory or inhibitory effect depends on the binding of anti-M3R autoantibodies to the different epitopes on M3R in SS. In other reports, anti-M3R autoantibodies in SS were shown to have multiple inhibitory effects on the smooth muscle contractions of the gastrointestinal tract including oesophagus, stomach, ileum, and colon to mediate a broader autonomic dysfunction [14,29]. A notable experiment conducted by Dawson et al. demonstrated that pSS IgG or known anti-M3R antibodies have an acute, reversible, inhibitory effect on the carbachol-induced M3R activity in primary human submandibular gland (hSMG) acinar cells [30]. Primary SS IgG further acutely inhibited Ca²⁺-activated K⁺ currents induced by muscarinic stimulation in human salivary gland (HSG) cells in a reversible manner [31]. Previously, Li et al. [16] demonstrated that carbachol-induced calcium mobilization was inhibited by long-term incubation of the cells for 12 h with anti-M3R autoantibodies. The mechanism underlying the chronic inhibitory effect of anti-M3R autoantibodies shown in HSG cells was further investigated in primary cultured hSMG epithelial cells [13]. In hSMG cells, M3R was internalized via clathrin-mediated endocytosis by incubation with pSS IgG for longer than 30 min (Figure 1). Therefore, the underlying mechanism of the inhibitory effect of pSS IgG on M3R function could be attributed to two consequent processes. First, M3R is acutely desensitized by direct occupation of agonist binding sites of the receptor by pSS IgG and phosphorylation of the receptor. Subsequently, M3R function is further decreased by clathrin-mediated receptor internalization in the continued presence of pSS IgG. After internalization of M3R, abundant pSS IgGs may bind to the remaining unoccupied M3Rs, thus resulting in a progressive and long-term loss of M3R function (Figure 2). It has been shown that aquaporin 5 (AQP5) trafficking is crucial in fluid secretion and is primarily regulated by M3R in salivary glands [32,33]. AQP5 trafficking to the apical membrane of rat parotid acinar cells was affected by the long-term (12 h) incubation of the cells with IgG from SS patients [16]. Recently, Lee et al. [34] further confirmed that AQP5 trafficking to the plasma membrane was inhibited by anti-M3R autoantibodies in SS. These findings strongly suggest that anti-M3R autoantibodies have multifaceted effects, possibly not only through direct binding of anti-M3R autoantibodies to M3R but also by affecting other molecules involved in salivary secretion.

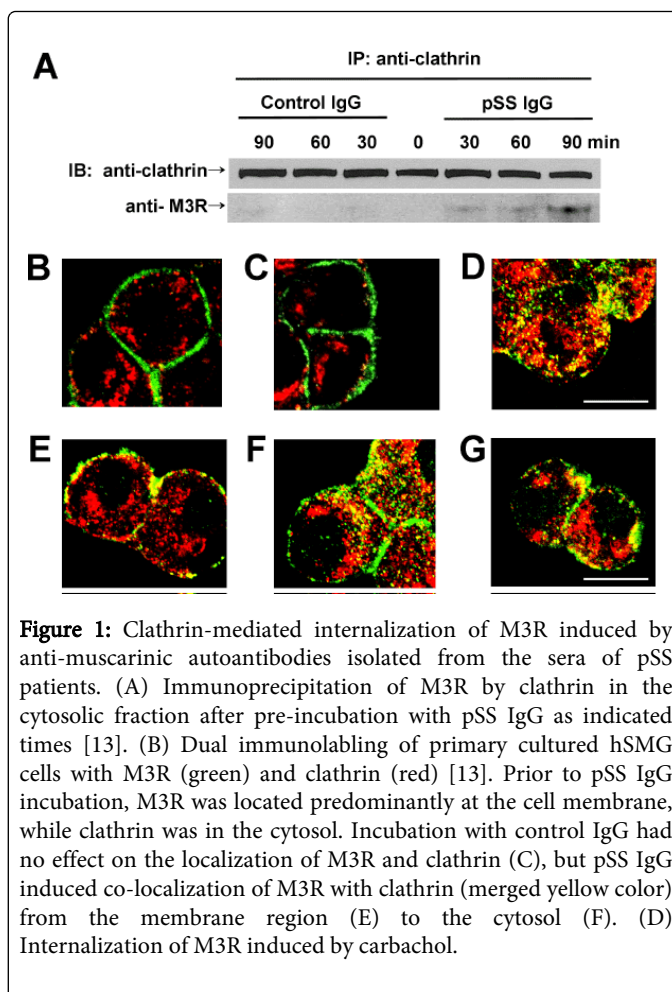


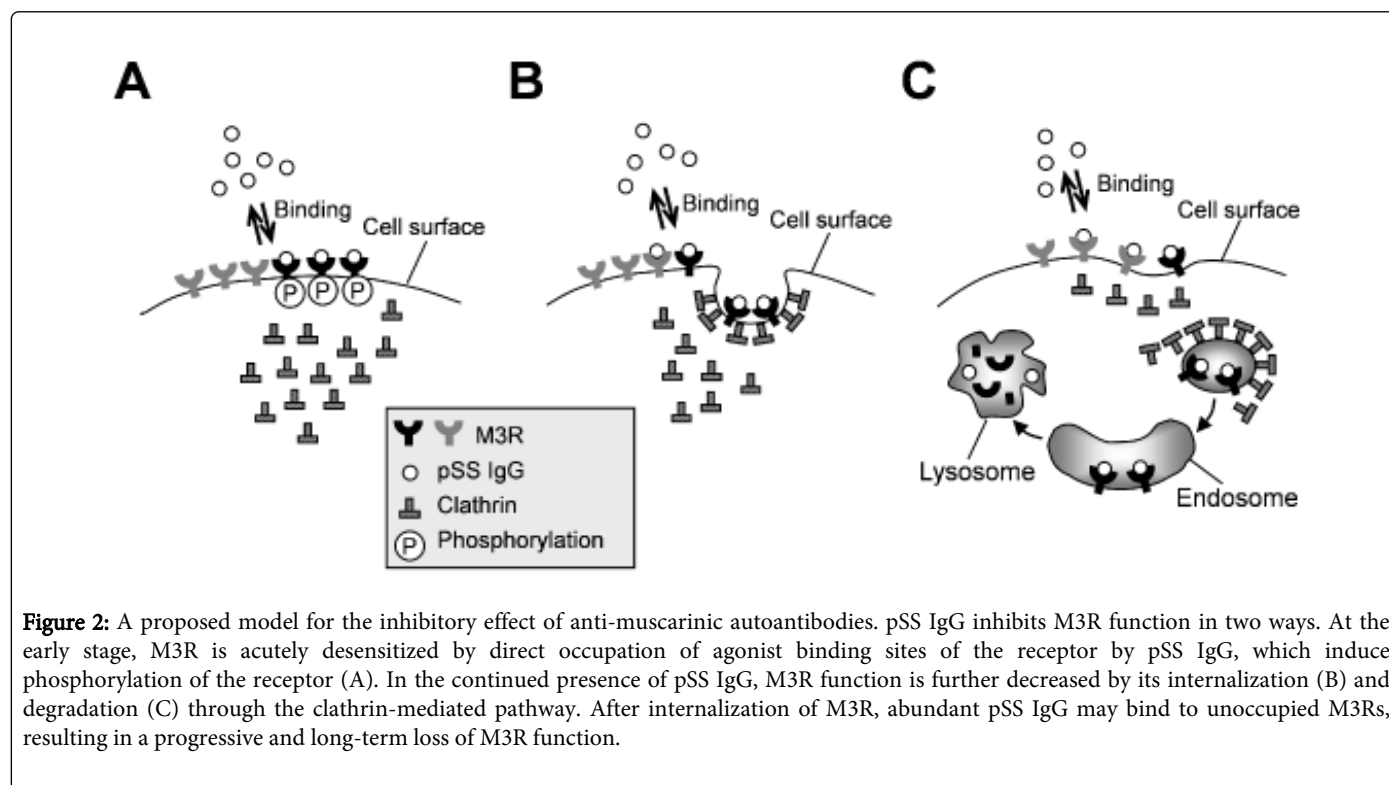
Figure 1: Clathrin-mediated internalization of M3R induced by anti-muscarinic autoantibodies isolated from the sera of pSS patients. (A) Immunoprecipitation of M3R by clathrin in the cytosolic fraction after pre-incubation with pSS IgG as indicated times [13]. (B) Dual immunolabeling of primary cultured hSMG cells with M3R (green) and clathrin (red) [13]. Prior to pSS IgG incubation, M3R was located predominantly at the cell membrane, while clathrin was in the cytosol. Incubation with control IgG had no effect on the localization of M3R and clathrin (C), but pSS IgG induced co-localization of M3R with clathrin (merged yellow color) from the membrane region (E) to the cytosol (F). (D) Internalization of M3R induced by carbachol.

Pathogenic Involvement of Anti-M3R Autoantibodies in SS

The prevalence of functional anti-M3R autoantibodies in patients with SS indicates a potential role of these autoantibodies in the pathogenesis and development of SS. Dawson et al. demonstrated that labial gland acinar cells isolated from patients with pSS were capable of functioning in intracellular calcium mobilization and that Ca²⁺-dependent K⁺ and Cl⁻ channels had a reduced sensitivity to ACh [35]. Nevertheless, as early as 2000, the muscarinic agonists, pilocarpine and cevimeline, were approved by the FDA to be a part of therapies for SS because of their partial effect on dryness of the eyes and mouth in a subpopulation of patients with 'moderate dryness' [36]. To directly prove the pathogenic role of anti-M3R autoantibodies in SS, Wang et al. [37] performed passive transfer experiments using BALB/c mice. They demonstrated that mice injected with IgG from SS patients having anti-M3R activity showed increased bladder detrusor contractibility in response to carbachol stimulation and a reduction in the bladder wall compliance compared to mice injected with IgG from healthy controls or SS patients lacking anti-M3R activity. The passive transfer of patient IgG also increased M3R expression in bladder detrusor, similar to up-regulation of M3R in labial salivary gland acini seen in primary SS [38]. A study performed by Lizuka et al. [39] demonstrated that Rag1^{-/-} mice inoculated by the splenocytes from M3R^{-/-} B6 mice, which were immunized with peptides derived from

the M3R extracellular regions, induced SS-like sialoadenitis. In M3R^{-/-}→Rag1^{-/-} mice, anti-M3R autoantibodies were detected in the sera of the mice. IFN-γ and IL-17-producing cells were also detected in the salivary glands of M3R^{-/-}→Rag1^{-/-} mice. Researchers further confirmed a crucial role of IFN-γ in the development of M3R-induced sialoadenitis (MIS) [40] as well as the involvement of IL-17 produced by M3R reactive T cells in the development of MIS [41]. Naito et al. found that altered peptides derived from the second extracellular domain of M3R inhibited IFN-γ production by suppressing the

number of IFN-γ-producing T cells in patients with SS [42]. A finding was also reported that leucopenia, a common extra-glandular manifestation seen in SS patients, was significantly more common in anti-M3R²¹³⁻²²⁸ positive pSS patients (53%) than in those without this antibody (14%) [21]. In an aforementioned report [26], anti-M3R²⁰⁵⁻²²⁰ positivity was associated with hematological abnormalities, such as leucopenia, anemia, and thrombocytopenia in pSS. These findings strongly suggest the involvement of circulating and systemic anti-M3R autoantibodies in the pathogenesis of SS.



Active Role of Salivary Gland Epithelial Cells (SGECs) in SS Autoimmunity

It has been noticed that salivary gland epithelial cells (SGEC) may have an active role in the immune response and pathogenesis of SS. SGECs adjacent to the sites of intense inflammation have been found to express high levels of various immunoinactive proteins, which are known to activate immune cells, including antigen-presenting molecules MHC class I and II [43], costimulatory molecules CD80/B7.1 and CD86/B7.2 [44], B-cell activating factor (BAFF) [45], adhesion molecules ICAM-1 [46], and various chemokines [47]. There are ectopic germinal center-like structures observed in the salivary glands of SS patients [48]. The active immunoreactive role might be started with direct cross-talk of SGECs to the environmental microbes surrounding it. Cultured SGECs derived from patients with pSS express Toll-like receptors (TLRs) including TLR-1, TLR-2, TLR-3, and TLR-4, which up-regulate the surface expression of ICAM-1, CD40, and MHC class I when they are activated [49]. The HSG cell line (ductal origin) can secrete cytokines such as TNFα and IL-6 when TLR-3 and TLR-4 are activated [50]. Taken together, it seems likely that SGECs may be capable of functioning as professional antigen presenting cells (APCs) such as macrophages, dendritic cells and B cells to present antigens or autoantigens to T cells. Recently, we investigated the possibility that M3R can be internalized by pSS IgG

incubation via both clathrin-dependent [13] and clathrin-independent endocytosis, in which marked down-regulation of MHC class I was observed (unpublished data). MHC class I expressed in all nucleated cells plays pivotal roles in both the innate and adaptive immune system by serving as a ligand of inhibitory killer cell immunoglobulin-like receptors (KIRs) on natural killer (NK) cells and by presenting intracellular antigens to cytotoxic T lymphocytes [51]. 'Missing self' due to loss of MHC class I molecules happens in viral infections [52]. Then, natural killer (NK) cells recognize the cells which are 'missing self' and kill the cells [53]. A decrease in the level of MHC class I at the plasma membrane of SGECs in our experiment (unpublished) may also not sufficiently induce subsequent adaptive immune response, resulting in an increased susceptibility to infection by intracellular pathogens. Thus, the interaction of M3R, a key molecule in salivary secretion, with MHC class I, one of the main molecules involved in the immune response, may provide an important clue for explaining a possible link between exocrinopathy and autoimmunity seen in SS.

Conclusion

The etiologies of SS are multifactorial and inconclusive. Salivary glands may have an intrinsic and active role in the abnormal immune response, although the initial step still remains unknown. M3R plays a key role in exocrine secretion. The presence of M3R reactive T cells,

the prevalence of anti-M3R autoantibodies in the sera, tears and saliva of patients with SS and the inhibitory effects of anti-M3R autoantibodies in various tissues strongly indicate that M3R-based interactions between SGEs and immune cells and the anti-M3R autoantibodies produced may play a pivotal role in the pathogenesis and development of SS. Further investigations are necessary to define this concept and may provide new insights and targets for SS therapy.

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