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Clinical associations of autoantibodies to human muscarinic acetylcholine receptor $3^{213–228}$ in primary Sjögren's syndrome

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Objectives. **The authors have previously identified a peptide of the human muscarinic acetylcholine receptor-3 (m3AChR) as a suitable antigen for the immunodetection of antimuscarinic acetylcholine receptor autoantibodies in primary Sjögren's syndrome (pSS). The aim of this study was to assess the clinical correlations and disease specificity of these antibodies.**
Methods. **Seventy-three pSS, 40 rheumatoid arthritis (RA), 19 systemic lupus erythematosus (SLE), 14 secondary Sjögren's syndrome (sSS) patients, 22 subjects in whom pSS was suspected but in whom the diagnosis not could eventually be established (suspSS) and 40 healthy subjects were investigated. An enzyme-linked immunosorbent assay system developed by the authors using a 16-mer peptide of the m3AChR (m3AChR$^{213–228}$) in a recombinant fusion peptide form was used as the antigen.**
Results. **Anti-m3AChR$^{213–228}$ antibody positivity was observed in 66 (90%) of the pSS patients. The antibody levels correlated positively with the number of extraglandular organ manifestations. Both the mean antibody levels and the occurrence of anti-m3AChR$^{213–228}$ positivity were significantly higher in pSS than in the comparison groups. The test discriminated the pSS patients from the various comparison groups with specificities of 65, 68, 71 and 50% for RA, SLE, sSS and suspSS, respectively.**
Conclusions. **The presence of m3AChR$^{213–228}$ antibodies is a common feature in pSS. Although it is significantly more common in pSS than in the comparison groups, anti-m3AChR$^{213–228}$ positivity is not exclusive to pSS.**

KEY WORDS: Antimuscarinic acetylcholine receptor-3 autoantibody, Disease specificity, Muscarinic acetylcholine receptor-$3^{213–228}$, Primary Sjögren's syndrome.

Sjögren's syndrome (SS) is a systemic autoimmune connective tissue disease characterized by the obligatory manifestations of keratoconjunctivitis sicca and xerostomia, and, in the majority of patients, by various other extraglandular organ involvements. The disease is classified as primary Sjögren's syndrome (pSS) if no other systemic autoimmune disease is present, and as secondary Sjögren's syndrome (sSS) if the symptoms of ocular and oral dryness evolve in a patient with an established diagnosis of another connective tissue disease, most commonly rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE).

A prominent feature of pSS is the presence of B-lymphocyte hyperactivity, leading to the presence of a variety of autoantibodies. In addition to the classical anti-SSA and anti-SSB antibodies, other novel antibodies have recently been detected. One of these is the autoantibody reactive with the muscarinic acetylcholine receptor subtype-3 (m3AChR) [1–4]. This receptor mediates the secretagogue cholinergic stimuli in the lachrymal and salivary glands, and studies on animal models of SS have indicated that antibodies from the sera of pSS patients reacting with the m3AChR are essential for the elicitation of a glandular dysfunction [2, 5].

We recently developed an enzyme-linked immunosorbent assay (ELISA) system for the detection of antibodies to a 16-mer peptide sequence of the human m3AChR (m3AChR$^{213–228}$ [6]). On application of this peptide sequence of the second extracellular loop (the ligand-binding region) of the human m3AChR, produced in recombinant form fused with glutathione-S-transferase (GST), the ELISA method differentiated the pSS patients from the healthy controls in a reliable and highly sensitive way. In the work presented here, we tested the sera of a larger cohort of pSS patients and of various comparison groups in order to determine the prevalence, clinical associations and disease specificity of m3AChR$^{213–228}$ antibodies.

Patients and methods

Study populations

Seventy-three pSS patients (70 females, mean age 55 yr, range 30–82) were included in this study. They all met the American–European classification criteria for pSS [7]. Five comparison groups were included: (group 1) 40 patients with RA (36 females, mean age 56 yr, range 27–80), classified according to the American College of Rheumatology (ACR) criteria for RA [8]; (group 2) 19 SLE patients (16 females, mean age 40 yr, range 26–73), fulfilling the ACR classification criteria for SLE [9]; (group 3) 14 sSS patients (all females, mean age 53 yr, range 32–63), who met the American–European classification criteria for sSS [7]; (group 4) 22 patients (21 females, mean age 52 yr, range 21–78) who were referred to our department because of suspected SS but in whom, after subsequent detailed examinations, the diagnosis of pSS could not be established (suspSS group); and

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(group 5) 40 healthy blood donors (all females, mean age 49 yr, range 23–62 yr). All of the patients in the suspSS group had either subjective plus objective xerophthalmia and xerostomia (a Schirmer test result of $\leq$5 mm/5 min and a Saxon test result of <2.7 ml/2 min), and/or a combination of other clinical features suggestive of pSS, including bilateral chronic parotid gland enlargement, purpura or arthralgia, with laboratory abnormalities including antinuclear antibody (ANA; 12 patients), anti-SSA (four patients) or anti-SSB (three patients) antibodies or rheumatoid factor (eight patients). The clinical diagnoses in this group included hypothyroidism, fibromyalgia, depression, hepatitis C virus-associated sicca complex or drug-induced xerostomia/xerophthalmia. Patients were not eligible for entry into group 1 or 2 if they had any signs or symptoms giving rise to the suspicion of sSS (oral or ocular dryness reported in response to specific questions, or abnormal Schirmer's or Saxon's test results). In the sSS group, the diagnoses associated with SS were RA ($n=7$), SLE ($n=5$) and systemic sclerosis ($n=2$). The study was approved by the Medical Ethics Committee of the University of Szeged. The authors declare that the patients gave informed consent to the examinations detailed in this work.

ELISA method

A recombinant protein containing the 16-mer peptide sequence KRTVPPGECFIQFLSE (KRSE, m3AChR$^{213–228}$) fused with GST (GST-KRSE) was produced in *Escherichia coli* as described previously [10, 11]. Microtitre plates were coated with 100 μl of 10 μg/ml GST-KRSE in 0.1 M Na_2CO_3 buffer, pH 9.6, containing 10 mM dithiothreitol overnight at 4°C. For every sample, GST alone was also coated in parallel with the GST-KRSE, and the quantities of antigens used for coating were equalized to the GST portion of the fusion partner. GST was therefore applied at a concentration of 9 μg/ml in the same coating buffer. Subsequently, the plates were washed with phosphate-buffered saline and blocked with SuperBlock Blocking Buffer (Pierce) for 1 h at room temperature. The plates were next washed, and the serum samples were added at a dilution of 1:100 in blocking solution, followed by incubation for 2 h at 37°C. After washing, incubation followed with horseradish peroxidase-labelled goat anti-human IgG antibody (1:10 000; Sigma) in blocking solution. After thorough washing, the reaction was developed with *o*-phenylenediamine in phosphate–citric acid buffer, pH 5.0, for 30 min at room temperature in the dark. The optical density (OD) values were measured at 492 nm. The peptide-specific OD value for each serum was calculated by subtracting the OD value of the GST from that of the GST-KRSE of the corresponding sample. The cut-off level between normal and positive values was taken as the mean + 2 S.D. in the healthy control group; this calculation resulted in a cut-off OD of 0.24.

Statistical methods

Correlations between the anti-m3AChR$^{213–228}$ antibody levels (quantified with the OD values) and continuous variables (e.g. age and disease duration) were assessed with Pearson's correlation test, while the Spearman signed rank test was used to investigate an association between the OD values and categorical variables (the presence or absence of organ manifestations or serological positivities). To investigate a correlation between the anti-m3AChR$^{213–228}$ levels and the number of extraglandular organ manifestations, the Jonckheere–Terpstra test was applied, as this was considered most appropriate for the testing of whether the distributions differ in a specified direction (i.e. whether an increasing number of organ manifestations is associated with higher amounts of anti-m3AChR$^{213–228}$ antibodies). Comparisons between the pSS group and the other patient groups were performed with an analysis of variance (ANOVA) test, with Dunnett's multiple comparison test as a *post hoc* test (differences between means), or with χ^2 tests (differences between the frequency distribution of occurrences).

Results

Sixty-six of the 73 pSS patients (90%) proved to be anti-m3AChR$^{213–228}$ antibody-positive. The various extraglandular organ manifestations and other clinical data, together with the serological abnormalities in the anti-m3AChR$^{213–228}$-positive and -negative groups and in the overall group of pSS patients are shown in Table 1. The antibody concentrations were not associated with the age of the patients or the disease duration, or with the severity of the glandular insufficiency, as assessed by the stimulated whole saliva production measured with the Saxon test. However, an increasing number of extraglandular organ manifestations in a given patient correlated positively with the concentration of anti-m3AChR$^{213–228}$ antibodies ($P<0.05$; Fig. 1). In fact, each of the organ involvements was more common in

TABLE 1. Demographic features and the presence of extraglandular organ manifestations and serological variables in anti-m3AChR$^{213–228}$-positive and anti-m3AChR$^{213–228}$-negative pSS patients and in the overall cohort

Clinical or serological variable	Anti-m3AChR$^{213–228}$-positive ($n=66$)	Anti-m3AChR$^{213–228}$-negative ($n=7$)	All ($n=73$)
Age (yr; mean)	55	60	55
Disease duration (yr; mean)	14	11	14
Saxon test (ml/2 min; mean)	1.11	1.97	1.20
Articular involvement	52 (79)	5 (71)	57 (78)
Raynaud's phenomenon	26 (39)	2 (29)	28 (38)
Vasculitis	15 (23)	0	15 (21)
Renal involvement	10 (15)	1 (14)	11 (15)
Lymphoma	5 (8)	0	5 (7)
Anaemia	15 (23)	1 (14)	16 (22)
Leucopenia[a]	35 (53)	1 (14)	36 (49)
ANA	49 (74)	5 (71)	54 (74)
Anti-SSA	50 (76)	4 (57)	54 (74)
Anti-SSB	33 (50)	3 (43)	36 (49)

Numbers indicate the numbers of patients involved, with the percentages in parentheses, unless otherwise stated.
Renal involvement: renal tubular acidosis with or without biopsy-proven chronic tubulointerstitial nephritis.
Vasculitis: palpable purpura or other, biopsy-proven skin vasculitic lesion.
Leucopenia was significantly more common in the anti-m3AChR$^{213–228}$-positive pSS patients than in those without this antibody.
[a]$P<0.05$.

the anti-m3AChR$^{213–228}$-positive patients than in those without this antibody, although this difference was statistically significant only in the case of leucopenia. As the m3 subtype of the AChR is also the functionally dominant receptor in the gastrointestinal and the urinary tracts [12, 13], we examined whether there is a relationship between the amount of anti-m3AChR$^{213–228}$ antibodies and the degree of parasympathetic dysfunction in these organs, as measured in our previous studies [14]. No correlation was found (data not shown).

The frequencies of anti-m3AChR$^{213–228}$ positivity in the five comparison groups are demonstrated in Table 2, together with the mean antibody concentrations. The distribution of the antibody levels is presented in Fig. 2. Both the mean antibody concentrations and the frequencies of anti-m3AChR$^{213–228}$ positivity were significantly lower in each of the comparison groups than in the pSS group. The sensitivity of the anti-m3AChR$^{213–228}$ measurement to pSS proved to be 90%. The specificity values are to be seen in Table 2. The anti-m3AChR$^{213–228}$ testing differentiates between the pSS and the various non-pSS patient groups with specificities varying in the interval 50–71%. The likelihood ratios (Table 2) indicate that a negative anti-m3AChR$^{213–228}$ result decreases the probability of pSS to 0.13–0.19 in the various patient comparison groups, while a positive test result leads to a 1.81- to 3.16-fold increase in the likelihood of pSS.

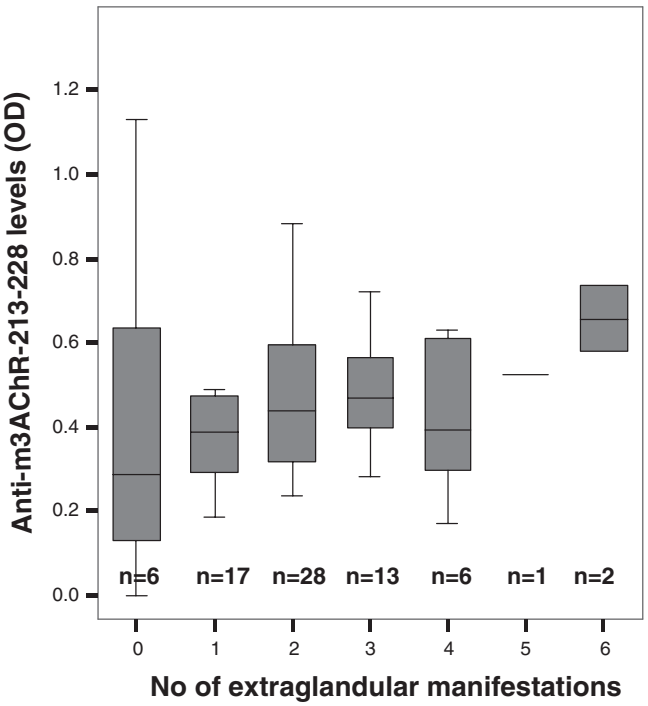

FIG. 1. Box and whisker plots representing anti-m3AChR$^{213–228}$ antibody levels in pSS patients with various numbers of extraglandular organ manifestations. An increasing number of extraglandular organ manifestations correlated positively with the mean anti-m3AChR$^{213–228}$ antibody titres ($P < 0.05$). Horizontal lines inside the box show median OD values; boundaries of the box show 25th and 75th percentiles; whiskers represent minimum and maximum values that are not extreme or outlier values. The numbers above the horizontal axis indicate the numbers of patients in each subgroup.

Discussion

In recent years, novel autoantibodies that react with the m3AChR have been identified in pSS. These antibodies have been demonstrated to play an essential role in the elicitation of the glandular dysfunction in the NOD mouse model of pSS [2, 5], possibly via binding to and exerting an inhibitory effect on the receptor [15, 16]. We recently developed an ELISA system which enables us to measure m3AChR$^{213–228}$ antibody levels on a large scale [6]. To our knowledge, this is the first report on the clinical associations of anti-m3AChR autoantibodies in pSS.

The data we obtained from a relatively numerous cohort of pSS patients indicate that these antibodies are present in the vast majority of pSS patients. There are some indications in our results that the presence or increasing concentrations of anti-m3AChR$^{213–228}$ antibodies may be associated with more extraglandular manifestations in the given patient, i.e. with a more 'systemic' form of pSS. In this regard, it may be interesting that, similarly to the present results, we previously found that each extraglandular organ involvement occurred with higher frequency in a subgroup of pSS patients who demonstrated an impaired microvascular response to cholinergic stimulation than in those in whom a normal response was observed [17]. However, as very few statistically significant differences have been revealed, this possibility needs further confirmation.

An intriguing question with regard to antimuscarinic autoantibodies is their *in vivo* role in the elicitation of the glandular dysfunction in pSS. We therefore tested whether there is a correlation between the level of anti-m3AChR$^{213–228}$ antibodies and the degree of salivary gland dysfunction, but we failed to demonstrate a direct relationship. However, in view of the high prevalence of anti-m3AChR$^{213–228}$ antibodies in pSS, together with the results of previous experiments [2, 4, 5, 17], and the physiological importance of the m3AChR in the regulation of the glandular function, we consider that the question of whether these autoantibodies play some role in the pathogenesis of pSS remains justified.

TABLE 2. Results of the anti-m3AChR$^{213–228}$ ELISA in the six groups examined, and the statistical parameters assessing the potential of the method to discriminate between pSS and the other comparison groups

	pSS ($n = 73$)	RA ($n = 40$)	SLE ($n = 19$)	sSS ($n = 14$)	suspSS ($n = 22$)	Healthy ($n = 40$)
Anti-m3AChR$^{213–228}$ levels (mean OD)	0.461	0.217*	0.164*	0.143*	0.250*	0.121¶
No. of anti-m3AChR$^{213–228}$-positive patients (%)	66 (90)	14 (35)¶	6 (32)¶	4 (29)¶	11 (50)*	0¶
Specificity		65%	68%	71%	50%	100%
Positive likelihood ratio		2.58	2.86	3.16	1.81	n.a.
Negative likelihood ratio		0.15	0.14	0.13	0.19	0.096

The mean OD values and the prevalences of anti-m3AChR$^{213–228}$ antibody positivity were significantly lower in each of the comparison groups than in the pSS patients.

n.a., not applicable.

*$P < 0.01$; ¶$P < 0.0001$.

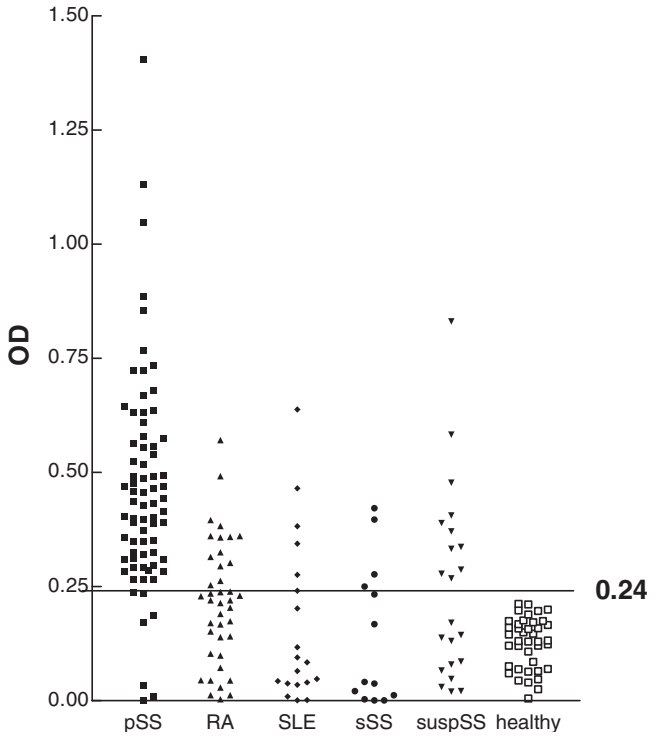

FIG. 2. Scattergram demonstrating the distribution of the OD values measured with the anti-m3AChR$^{213–228}$ ELISA in the different study groups. The cut-off value is indicated by the horizontal line. The number of patients with positive test results and the statistical comparison between the groups are shown in Table 2.

The pSS group was significantly different from each of the comparison groups in terms of both the frequency of anti-m3AChR$^{213–228}$ positivity and the mean anti-m3AChR$^{213–228}$ levels. However, the potential of anti-m3AChR$^{213–228}$ antibodies for clinical discrimination between pSS and the examined comparison groups of patients with the clinical conditions that most commonly cause differential diagnostic difficulties in clinical practice proved inappropriately low in the present setting. Despite the statistically highly significant differences in the various groups, the number of anti-m3AChR$^{213–228}$-positive patients was sufficiently high in all the comparison groups to give the test modest specificity. An analogous approach to the assessment of the clinical value of the test is the calculation of likelihood ratios. This indicates that a negative test result is relatively strong evidence against the diagnosis of pSS, while a positive test result itself is not sufficiently helpful. This is still true even if we consider that the members of the suspSS group were selected in a fairly strict way. These patients exhibited many features resembling pSS, including not only a subjective, but also an objective glandular dysfunction, compatible in severity with that required for the diagnosis of pSS in the American–European classification criteria [7]. Many of them were ANA-positive and some of them also had anti-SSA/SSB antibodies. However, none of them could be considered to have pSS, either as concerns the number of classification criteria or by clinical judgement.

The marked difference between the pSS and sSS groups with regard to anti-m3AChR$^{213–228}$ antibodies may support the view that these are diseases with different pathology and pathogenesis [18]. However, with regard to the data in the literature, this finding requires confirmation. The presence of antibodies to the human m3AChR in an evaluable number of patients with diseases other than pSS has been reported in only one publication [3]. In that study, 14 of 17 sSS patients were found to be anti-m3AchR-positive. A further difference between the results of that study and ours is that the prevalence of anti-m3AChR antibodies in a control group termed 'non-SS dry eye' patients was very low. In addition to the fact that a different (although partly overlapping) sequence of the human m3AChR was used as antigen, tested with a different method (synthetic peptide ELISA), the control patient selection was probably also different from ours, as evident from the above considerations about the suspSS group. The involvement of more comparison patients and more specific determination of the control groups may help answer the questions raised by the present preliminary study concerning the disease-specificity of anti-m3AChR antibodies.

In conclusion, we have revealed a high prevalence of anti-m3AChR$^{213–228}$ antibodies in pSS. In our opinion, an attempt should be made to increase the specificity of the applied ELISA test in order to assess whether anti-m3AChR testing may be of clinical benefit as a differential diagnostic aid in pSS.

Rheumatology	Key messages
	• Antimuscarinic acetylcholine receptor-3$^{213–228}$ autoantibodies are highly prevalent in primary Sjögren's syndrome. • Mean levels and occurrences are higher than in rheumatoid arthritis, systemic lupus erythematosus and secondary Sjögren's syndrome.

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