Role of M₃ Muscarinic Acetylcholine Receptor Antibodies as a New Marker in Primary Sjögren Syndrome

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Abstract

Aims: This paper investigates the presence of M₃ muscarinic acetylcholine receptor autoantibody present in the serum of patients with primary Sjögren syndrome (pSS). Main methods: We detected the levels of M₃ mAChR peptide IgG, PGE₂, IL-1β in serum of SS patients using the enzyme-linked immune sorbent assay (ELISA). To measure the quantity of nitrite/nitrate, we used Griess reagent system. Key findings: Titres of M₃ mAChR antibody in sera from SS patients are significantly enhanced compared to healthy subjects (control). The enhancement of these autoantibodies is accompanied by the increase of the levels of PGE₂, IL-1β and nitrite/nitrate in serum. Under in vitro conditions, the synthetic human M₃ peptide impaires the increment of M₃ mAChR antibody but not that of nati-Ro/SSA antibody. In positive anti-Ro/SSA antibody patients, the increment of M₃ mAChR peptide IgG and the measured pro-inflammatory substances is related. Significance: On this basis, anti M₃mAChR peptide IgG can be said to act as a modulator of the immune system and to play a role in the host-chronic increment of proinflammatory substances in SS patients with positive Ro/SSA antibody. This association between the antibody and the pathogenesis of SS disease may result in useful predicting SS.

Keywords

Anti-Ro/SSA Antibody, Anti M₃mAChR Peptide IgG, IL-1β, PGE₂, Nitric Oxide

1. Introduction

Sjögren’s syndrome (SS) is a systemic autoimmune disease primarily affecting
the exocrine glands. Although clinically characterized by dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia), a series of extra-glandular manifestations can also develop [1]. The disease occurs either in a primary form (pSS) or in association with other autoimmune diseases (associated form, aSS) such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis (SSc). SS affects mostly women, represented by the ratio of 9 female to 1 male and a prevalence in the range of 0.1% to 4.8%, depending on the population examined and the classification criteria used [2] [3] [4] [5].

The presence of serum autoantibodies in the course of SS is of key significance. They are therefore usually used as biomarkers for the classification and diagnosis of pSS and aSS [4] [5]. However, other autoantibodies not currently used in clinical practice such as anti-M₃ muscarinic acetylcholine receptor (anti-M₃mAChR), have also been described in pSS/aSS [6] [7] [8] as useful markers [9] [10].

It is known that anti-Ro/SSA and anti-La/SSB antibodies are directed against ribonucleoprotein complexes [11]. Their localization is mainly cytoplasmic [12] and the increased expression of Ro52 in SS and SLE patients is probably implicated positively or negatively in the alteration of cellular immune responses and in the increased apoptosis observed in pSS patients [13] [14]. Ro52 in pSS can act as a negative regulator of interferon production, ubiquitination and degradation of the transcription factors IRF-3 [15], IRF-7 [16] and IRF-8 inducing major alterations in cytokine expression and production [17]. There is a higher prevalence of extra-glandular clinical manifestations in pSS/aSS anti-Ro/anti-La antibody positive patients [5] [18]. In patient with pSS, the presence of these antibodies increases the risk of neonatal congenital heart block [19] or neonatal SLE [20].

One of the remaining unsolved questions in pSS is the relationship between anti-Ro/SSA and anti-M₃mAChR antibodies and their role in the production of pro-inflammatory cytokines (IL-1β/PGE₂-nitric oxide) in the pathogenesis of pSS. Against this background, the aim of the present work is to analyze the distribution of positive or negative serum anti-Ro and anti-M₃mAChR antibodies in a population of pSS patients. In addition, we investigate the relation between the production of IL-1β, PGE₂ and nitric oxide (detected by nitrate/nitrite production) and if the presence of these autoantibodies is in view of finding out whether they are able to alter or participate in the pathogenesis of SS disease.

2. Material and Methods

2.1. Patients

The subjects of this study were 26 pSS patients (14 anti-Ro/SSA positive, 12 anti-Ro/SSA negative) and 24 healthy volunteers all female, (age 39 - 54 years) selected from the metropolitan area of Buenos Aires (Table 1). The diagnosis of pSS fulfilled the criteria described by Vitali et al. [21] and was given by means of a positive biopsy with a score focus of 3.8 ± 0.07. Anti-Ro/SSA positivity or anti-Ro/SSA negativity was determined by enzyme-linked immunosorbent assay (ELISA).
Table 1. Characteristics of the study populations.

<table>
<thead>
<tr>
<th>Demographics Factors</th>
<th>pSS Patients (n = 26)</th>
<th>Healthy Subjects (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Ro (+)</td>
<td>Anti-Ro (−)</td>
</tr>
<tr>
<td>Gender Male Female</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Age, mean ± SD years</td>
<td>39.2 ± 12.9</td>
<td>48.6 ± 14.5</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Xerophthalmia</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>ANA positive</td>
<td>14 (100)</td>
<td>2 (16)</td>
</tr>
<tr>
<td>Anti-Ro/SSA positive antibodies, %</td>
<td>14 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-M₃mAChR positive antibody, %</td>
<td>14 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-M₃mAChR peptide IgG, mean ± SEM</td>
<td>1,450 ± 0.12*</td>
<td>0.077 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Values in parenthesis are the percentages. Anti-M₃mAChR peptide IgG: *P < 0.001 between anti-M₃mAChR peptide IgG anti-Ro (+) and anti-Ro (−) patients and healthy subjects. ND: not detected. ANA: antinuclear antibody. Clinical state of patients with pSS: + leve, ++++ severe.

The protocol was approved by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina) and complied with the tenets of the Declaration of Helsinki. All subjects signed a written informed consent.

2.2. Serological Studies

Anti-Ro-SSA procedure: Saline-soluble extractable nuclear antigens (ENA) were obtained from human spleen in phosphate buffered saline (PBS) for anti-Ro. Patient sera were tested undiluted and diffusion was carried out at room temperature in a humidified chamber for 48 hours. Precipitin lines were identified by comparison with reference sera. ELISAs for total anti-Ro (60kD and 52kD Ro-proteins) was performed with a commercial Kit based on purified antigens (OrgentecDiagnostika, Mainz, Germany) and the assays were carried out according to the manufacturer’s protocols on an automated ELISA instrument (Radim, Pomezia RM, Italy). Values greater than 25 UI/ml were considered positive.

Purification of human IgG: The serum IgG fraction from patients with pSS and from normal individuals (control) was isolated using protein G affinity chromatography as described elsewhere [9]. Briefly, sera were loaded onto the protein G affinity column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with 1 M Tris-HCl (pH 8.0) and the columns were washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine-HCl, pH 3.0, and immediately neutralized. The concentration and purification of IgG were determined using a radial immunodiffusion assay.

Anti-M₃ peptide IgG procedure: The IgG fraction from 26 patients with pSS and 24 healthy subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AfiGel 15 gel (Bio-Rad, Richmond, CA,USA) as described by Reina et al. [9]. Briefly, the IgG fraction was loaded onto the affinity column equilibrated with PBS. The non-peptide fraction was first eluted with the same buffer. Specific anti-peptide antibodies
were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies was determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic M3 receptor peptide was evaluated by ELISA. The concentration of the affinity-purified anti-M3 peptide IgG (1 × 10⁻⁷ M) increased optical density (mean ± SEM, 2.4 ± 0.2). The non-anti-M3 peptide IgG fraction from the column showed OD values (0.27 ± 0.06) similar to those of normal IgG from healthy individuals taken as control (0.26 ± 0.05). The normal IgG fraction purified by affinity column chromatography gave a negative result (0.30 ± 0.03). ELISA was performed as described previously [6].

2.3. IL-1β and PGE₂ Procedures

Serum IL-1β and PGE₂ were measured by ELISA, carried out according to the manufacturer’s protocols (IL-1β Enzyme Immune Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA; PGE₂Biotrack Enzyme Immune Assay System, Amersham Bioscience, Piscataway, NJ, USA). Normal values of IL-1β range from 0.2 to 5.8 pg/ml, with an average of 3.2 ± 1.4 pg/ml in healthy individuals. The optical density (OD) cutoff value of PGE₂ was 4.4 ± 0.33 ng/ml and that of IL-1β 2.0 ± 0.02 pg/ml. All serum samples were frozen promptly after collection and kept at −80°C until used for PGE₂ and IL-1β and determinations. The PGE₂ and IL-1β results are expressed as ng/ml and pg/ml, respectively.

2.4. Nitrate and Nitrites Assay

The 20 μl serum samples were mixed with an equal volume of 100 μl of Griess reagent (Ingredient A: 0.1% naphthalene diamine dihydrochloride at a final concentration of 5 mmol/l; Ingredient B: 1% sulfanilamide at a final concentration of 5 mmol/l in orthophosphoric acid) in a 96 well microtiter plate (NUNC, Roskilde, Denmark). Nitric oxide concentrations were determined by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co, Ann Arbor, MI, USA) and measured spectrophotometrically at 540 nm using a microplate reader (Reader Model 230 S; OrganonTeknika, Boxtel, The Netherlands) following the criteria of Green et al. [22]. The nitrate and nitrites values are expressed as μM/ml.

2.5. Statistical Analysis

The statistical significance of the difference between the groups was determined by Student’s 2-tailed t-test for unpaired data. ANOVA test and Student-Newman-Keuls test were also performed when pair-wise multiple comparisons were necessary. In all cases, P values less than 0.05 were considered significant.

3. Results

Table 1 summarizes the clinical and laboratory characteristics of all patients studied with pSS Ro (+), Ro (−) and healthy subjects (control) based on demographics data and the values of detected serum antibodies.
To demonstrate the presence of serum IgG directed against M₃mAChR antibodies, we performed an ELISA assay. The coating antigen used for this purpose was a M₃ human synthetic peptide corresponding to the amino acid sequence of the second extracellular loop (K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I) of human glandular M₃mAChR.

The scatter gram of Figure 1 shows the optical density values (OD) of each of the 14 Ro-positive [Ro (+)] pSS patients and 12 Ro-negative [Ro (−)] pSS patients. The OD values obtained with the reactive autoantibodies were always more than two (2) standard deviation (SD) higher than those of healthy subjects, who were negative as Ro (−) patients in this study system.

It also shows that a significantly higher levels of anti-M₃mAChR peptide IgG (P < 0.001) was present in Ro(+) patients sera as compared to that of Ro(−) pSS patients. This difference was not statistically significant in the case of healthy subjects as compared to pSS Ro(−) patients whereas it was significant between healthy subjects and pSS Ro(+) ones (P < 0.001).

Figure 2 shows that serum IL-1β levels are significantly elevated in patients with pSSRo(+) as compared to those of healthy subjects and pSS Ro(−) patients (P < 0.001). Neither the cytokine levels in patients with SS Ro(−) nor in healthy subjects were statistically significant.

**Figure 1.** Scatterogram showing the immunoreactivity of circulating IgG against M₃ synthetic peptide IgG in 14 patients with anti-Ro(+), 12 patients with anti-Ro(−) and 24 healthy subjects used as control; individual OD values (1/30 dilution); OD cutoff value of 0.30 ± 0.03 anti-M₃mAChR peptide IgG; P < 0.001 between anti-Ro(+) versus healthy subject and anti-Ro(−); non-significant differences between anti-Ro(−) and healthy subjects.
Figure 2. Scatterogram showing the levels of IL-1β in serum of patients with pSS; values for each serum sample (1/30 dilution) from 14 pSS Ro(+), 12 pSS Ro(−) and 24 healthy subjects; IL-1β cutoff value 2.60 ± 0.14; P < 0.001 between anti-Ro(+) patients versus Ro(−) and healthy subjects; no significant differences between patients with SS Ro(−) and healthy subjects.

Figure 3 shows the values of PGE₂ in Ro(+) versus Ro(−) pSS patients. It can be seen that the serum levels of PGE₂ did not differ significantly between the two study groups and are significantly elevated compared to healthy subjects (P < 0.001).

The NO levels in patients with pSS were significantly higher than those in healthy subjects (Figure 4). Mean nitrate/nitrite concentrations in patients with pSS were 27.8 ± 2.3 (P < 0.001) µM while those in healthy subjects and Ro(−) patients were significantly lower, rating 12.1 ± 4.6 and 10.8 ± 3.1 µM (P < 0.0001) respectively.

It should be noticed that Ro(+) pSS patients were found to have high levels of anti-M₃ mAChR peptide IgG accompanied with an increased serum level of IL-1β, PGE₂ and NO, although the level of PGE₂ and NO observed in Ro(+) or Ro(−) pSS patients remain the same.

4. Discussion

In this study we demonstrate that the presence of anti-Ro(+) antibodies in the sera of pSS patients is associated with an increased serum concentration of anti-M₃ mAChR antibodies.

Functional studies reveal in addition that the presence of anti-Ro(+) antibodies and anti-M₃ mAChR antibodies is accompanied by increased levels of serum IL-1β, PGE₂ and NO. The immune cytokine IL-1β could be, in part, responsible for the increment of the levels of the pro-inflammatory mediators such as, PGE₂.
Figure 3. Scatterogram showing the levels of PGE$_2$ in anti-Ro(+) and anti-Ro(−) pSS patients; individual values for each serum sample (1/30 dilution) from 14 pSS Ro(+), 12 pSS Ro(−) and 24 healthy subjects; PGE$_2$ cutoff value 0.40 ± 0.01; P < 0.001 between anti-Ro(+) and anti-Ro(−) versus healthy subjects; no significant difference between SS Ro(+) and SS Ro(−) patients.

Figure 4. Histogram showing a comparison between NO expressed as nitrates/nitrites (µM/ml) concentration in serum of pSS patients Ro(+), pSS patients Ro(−) and healthy subjects used as controls. Mean ± SEM of 14, 12 and 24 studied individuals respectively in each case; *P < 0.001 versus healthy subjects.
and NO. These in turn, contribute to the severity of the inflammatory reaction occurring at the glandular and/or extraglandular sites.

It is important to note that one of the characteristic features of pSS is the presence of autoantibodies against Ro/SSA antigen. The role of this antibody in the pathogenesis of SS is generally considered as a nonspecific molecule that becomes a target in pSS, possibly as a modulator of the altered innate immunity [23]. However anti-M₃mAChR autoantibodies, which are organ-specific, could also be a specific factor participating in the pathogenesis of SS [13].

Based on the above, it could be speculated that viral infection of salivary gland epithelial cells leads to over expression of Ro 52 (a metabolic product of the anti-Ro antibody) as a defensive mechanism both to suppress viral replication and to protect the host from activation of the INF system. It could also be said that INF drives glandular inflammation accompanied by cellular lysis and apoptosis [13] [24]. Furthermore the Ro can be said to cause oxidative damage to glandular epithelial cells due to its capacity to stimulate proinflammatory cytokines such as IL-1β and PGE₂, leading to the maintenance of glandular tissue inflammation. All of these changes are also observed in the course of SLE [25].

Since increased levels of anti-M₃mAChR antibodies are found to have prevalence in Ro(+) pSS patients and this antibody is organ specific, it may be reasonably considered that its increment may be implicated in etiopathology of the disease. This is the case since anti-M₃mAChR IgG binds to, and activates, the mAChR subtype M₃ of the salivary gland. This produces xerostomia and glandular parasympathetic dysfunction, which in the chronic course of the disease, results also in severe extraglandular manifestations [9] [10] [11].

At this point of the discussion, anti Ro(+) and anti M₃mAChR antibodies could be considered useful both in the diagnosis and prognosis of pSS. They could also help to identify more active patients in whom the clinical behaviour is not clear and in this way serve as a new biomarker.

Our results are supported by recent studies describing an association between prolonged QTc interval [26], and the increment of anti-Ro antibodies in connective tissue disease (CTD) [27] [28]. Studies using human cardiac tissues showed that IL-1β affects the myocardium and treatment with IL-1β for a few hours decrease heart contractility [29]. It is important to remark that the relevance between circulating IgG against M₃ synthetic peptide IgG and levels of IL-1β could indicate some participation in the prolonged QTc intervals that occurs in connective tissue diseases [27] [28].

The fact is that the breakdown of self-tolerance in the activation of CD4+ T helper cells and B cells disturbances occurring in the course of pSS is not enough to maintain the disease. Some organ-specific new protein (could be a glandular M₃mAChR autoantibody) is required as a new component to spread the antigen and cause the parasympathetic dysfunction with all the sicca symptoms.

5. Conclusion

In summary, we have found enhanced titres of anti-Ro(+) and anti M₃mAChR
peptide IgG in patients with pSS as well as a high level of IL-1β, PGE₂ and NO. This may be related to the time of the evolution of the disease or the active or non-active form of the disease. As IL-1β affects the equilibrium of the immune system, we consider that this cytokine initiates the process which culminates with the increment of the levels of proinflammatory substances such as PGE₂ and NO together with those of anti-M₃mAChR peptide IgG. All of these events contribute to the pathogenesis of this syndrome.

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References


