Serum Interleukins Nitric Oxide Anti-Ro(+) and Anti-M₃ Muscarinic Acetylcholine Receptor Auto Antibodies Levels in Patients with Primary Sjögren’ Syndrome

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Abstract

Background
A variety of interleukins producing by T-helper cells have been linked to pathogenesis of primary Sjögren’s syndrome. In this study we tested the IL-1β, IL-6, IL-10 and IL-17 levels as well as the levels of nitric oxide and anti-Ro(+) and anti-M₃ muscarinic acetylcholine receptor auto antibodies, as an intricate and complex mechanism in Sjögren’s syndrome with parasympathetic dysfunction of exocrine glands.

Methods
Serum levels of all interleukins, nitric oxide and auto antibodies were detected by ELISA assay and were compared with the values obtained in healthy individuals taken as controls.

Results
Serum concentrations of cytokines were significantly higher in patients with primary Sjögren’s syndrome (pSS) as compared with those obtained in healthy individuals taken as controls. The nitric oxide (NO) levels and the anti-Ro(+) and anti-M₃ muscarinic acetylcholine receptor (mAChR) levels were elevated significantly in pSS patients than those of healthy individuals.

Conclusion
pSS patients showed increased levels of macrophages and lymphocyte-derived cytokines and NO indicating the existence of an immune activation state together with the presence of anti-Ro(+) and anti-M₃ mAChR auto antibodies, could participate in the Pathophysiology of the pSS markers of disease as a responsible of the clinical symptoms and signs and poor quality of life in patients with SS disease.

Keywords
Interleukins; Nitric Oxide; Anti-Ro/SSA Antibody; Anti-M₃ MACHR Antibody

Introduction
Primary Sjögren’s syndrome (pSS) is an autoimmune disease characterized by the lymphocytic infiltration to lachrymal and salivary glands conducing to an impaired secretory activity leading to the most important manifestations of the disease, keratoconjunctivitis sicca [1] and xeroslomia [2].

B cell hyper reactivity is manifested by the presence of hyper gamma globulinemia and auto antibodies mainly

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against ribonucleoproteins (SSA/Ro and SSB/La) [3] and M₃ muscarinic acetylcholine receptors (M₃ mAChR) [4].

Cytokines, such as interleukin-1β (IL-1β) [5] and anti-inflammatory mediators such as prostaglandins E₂ (PGE₂) [6], metalloproteinase C (MPP-3) [4] and nitric oxide (NO) [7] have been found increased levels in pSS patients suggesting an important role in the pathogenesis of the disease [8, 9].

In the Pathophysiology of Sjögren’s syndrome, T and B cells infiltrate the salivary and lacrimal glands. As a consequence of the destruction of glandular cells by cytotoxic T cells, production of cytokines and auto antibodies inhibiting glandular function, the production of saliva and tears is decreased. The feeling of dry eyes and mouth is frequently not noticed by the patients. Therefore, Sjögren's syndrome should also be considered when extraglandular manifestations such as vasculitis, polyneuropathy or arthritis or lupus erythematosus occur, even when the patients do not complain of dry eyes and mouth [10].

The aim of the present work was to analyze and tested the IL-1β, IL-6, IL-10 and IL-17 levels as well as the levels of nitric oxide (NO) as possible cytokines-induced inflammatory mediators in primary Sjögren’s syndrome (pSS) and anti-Ro(+) and anti-M₃ mAChR autoantibodies.

Materials and Methods

1. Ethical Approval of the Study Protocol

The study protocol complied with the tenets of the Declaration of Helsinki and accomplished with the rules established by the Ethics Committee of the University of Buenos Aires (Buenos Aires, Argentina). All subjects provided written informed consent.

2. Patients

The subjects of this study were 14 pSS patient anti-Ro/SSA positive and 14 healthy volunteers all female, (age 39-54 years) selected from the metropolitan area of Buenos Aires. The diagnosis of pSS fulfilled the criteria described by Vitali et al. [11] and was given by means of a positive biopsy with a score focus of 3.8±0.07. Anti-Ro/SSA positivity was determined by enzyme-linked immunosorbent assay (ELISA).

3. Serological Studies

3.1 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 (Orgentec Diagnostika, 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 22 IU/ml were considered positive.

3.2 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 [4]. 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.

3.3 Anti- M₃ Peptide IgG Procedure

The IgG fraction from 14 patients with pSS and 14 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 elsewhere [7] 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 (1x10⁻⁷ 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 [4].

4. Interleukin Determination

A blood sample was taken from each patient and control and sera were stored at −80°C until they were tested for cytokine concentrations. A sandwich immunoassay-based protein array system (Biosource International, Camarillo, CA, USA), which contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein, was used to determine the serum concentration of IL-1β, IL-6, IL-10 and IL-17. A broad range of standards ranging from 1.95 to 32000 pg/ml was used for quantifying the wide range of concentrations of these five cytokines, and to provide the greatest sensitivity. This captured immunoassay was then read by the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA, USA), which uses Luminex fluorescent-bead-based technology (Luminex Corporation Austin, TX, USA). The results were expressed as pg/ml.

5. Nitrate/nitrites Determination

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 230S; Organon Teknika, Boxtel, The Netherlands) following the criteria of Green et al.12 The nitrate and nitrites values are expressed as μM/ml.

6. Statistical Analysis

Student’s t-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and the Student–Newman–Keuls test were employed when pair-wise multiple comparison procedures were necessary. Differences between mean values were considered significant at P < 0.05.

Results

ELISA assays were carried out to determine the levels of anti Ro (+) and anti M3 mAChR autoantibodies in the serum of pSS patients and compared this values in healthy individuals used as controls. Table 1 shows the optical density (OD) values for serum from pSS patient that were significant greater (P < 0.0001) than those observed in control group. These results confirmed previous work done in our laboratory were SS patients present both auto antibodies.

<table>
<thead>
<tr>
<th>Serum autoantibodies</th>
<th>Primary Sjögren’ syndrome patients</th>
<th>Healthy individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ro (+)</td>
<td>28 UI/ml</td>
<td>0</td>
</tr>
<tr>
<td>Anti-M3 mAChR</td>
<td>2.12 ± 0.18</td>
<td>0.26 ± 0.05</td>
</tr>
</tbody>
</table>

Values expressed represent the media ± SEM of 15 patients with pSS and 15 healthy individuals in each case P < 0.001. Values are expressed as international units per millilitre and optical density by ELISA respectively.

Serum interleukins studied in the present work achieved levels significantly higher in pSS patients as compared to the healthy individuals. It is important to note that the level of IL-10 was the highest compared with the others kind of cytokines.

By the other hand, Table 2 also shows that the nitric oxide levels expressed as nitrite/nitrate in patients with pSS was significantly higher than those in healthy individuals taken as controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>pSS patients (n=15)</th>
<th>Healthy individuals (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>24 ± 9.2 pg/ml</td>
<td>0</td>
</tr>
<tr>
<td>IL-6</td>
<td>18 ± 8.9 pg/ml</td>
<td>0</td>
</tr>
<tr>
<td>IL-10</td>
<td>44 ± 9.9 pg/ml</td>
<td>5 ± 2.8 pg/ml</td>
</tr>
<tr>
<td>IL-17</td>
<td>14 ± 9.1 pg/ml</td>
<td>0.2 ± 0.2 pg/ml</td>
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<tr>
<td>Nitrates/nitrites</td>
<td>33 ± 4.3 pg/ml</td>
<td>16 ± 5.2 pg/ml</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of n = Number of Serum tested for each patient and healthy individual.

The possible association between the elevated cytokines levels determined in the present studied and the elevated levels of No, could be and important factor that occurs in the pSS disease and may be are related to the Pathophysiology of this disease as response to the inflammation, like seen in autoimmune diseases.
Table 3 shows the clinical characteristics of our population of pSS patients in relationship to the most frequently signs of the disease. The xerostomia, xerophthalmia and xerosis were the most prevalent in our studied pSS patients, which fatigue and Raynoud syndrome are the less frequently in our population studied.

### Table 3: Clinical Characteristics of pSS Patients and Healthy Individuals

<table>
<thead>
<tr>
<th>Patients</th>
<th>xerostomia</th>
<th>xerophthalmia</th>
<th>xerosis</th>
<th>fatigue</th>
<th>Raynoud syndrome</th>
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Clinical sing features in all pSS patients studied (x: leve; xx or xxx: Moderate; xxxx Severe)

### Discussion

This study demonstrated elevated cytokines and NO levels in the circulation of the patients with pSS which is in agreement with previously reported data [13, 14]. The increment of cytokines levels and NO levels in pSS patients are most probably a reflection of the systemic response to the inflammation that occurs in autoimmune diseases. Similarly, the role of IL-1β and IL-17 as a proinflammatory mediators from monocytes and peripheral blood mononuclear cells [15] with the increment in the NO levels, acting as the additive and synergistic effects in inducing the pathology that have been described in pSS. Concomitantly, with the presence of anti Ro(+) and anti M₃ mAChR auto antibodies, that expressed a cardinal signs of pSS [5, 16].

The increment in interleukins levels are related with health-related quality of life in a population of pSS patients. It was demonstrated that reduced quality of life of pSS patients is correlated with the increment of serum level of proinflammatory interleukins [17]. The fatigue in pSS patients is accompanied by IL-6 increment that also may be involved in the generation of pain and hyperalgesia in pSS patients as an endocrine action [18-20]. On the other hand, IL-10 has a predominance of the immune action in pSS patients [18, 19].

In addition both interleukins cited above, are able to acts as a modulator of a complex signalling cascade and secondary production of nitric oxide (NO) [18] and prostaglandins (PGE2), [6] which are both present in the course of autoimmunity in pSS patients.

IL-10 plays a role in pSS disease characterized by lymphocytic infiltration of salivary and lacrimal glands by promoting B-cell activation and auto antibodies (anti-Ro(+)) and anti-M₃ mAChR) production [21].

Serum concentrations of IL-17 were significantly higher in patients with pSS as compared to those obtained in healthy individuals as well as the levels of NO as possible IL-17-inducer product. This is a complex mechanism that could occur in autoimmune disease with disorder of parasympathetic system in exocrine glands in pSS patients. Taken into account that the increase levels of IL-17 and its ability to increase NO levels and anti-Ro(+) and anti-M3 mAChR auto antibodies, could implicate a cytotoxic injury in exocrine glands in the acute course of...
SS, also all together has been linked to pathogenesis of SS that autoimmunity and inflammation take place [22, 23].

Conclusion
pSS patients showed increased levels of macrophages and lymphocyte-derived interleukins and NO indicating the existence of an immune activation state together with the presence of anti-Ro(+) and anti-M3 mAChR autoantibodies. Both, could participate in the pathophysiology of the pSS markers of disease as a responsible of the clinical symptoms and signs and poor quality of life in patients with SS disease.

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References
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