Abstract

Aims

This paper investigates the action of M1 muscarinic acetylcholine receptor antibody present in serum from patients with Sjögren syndrome (SS).

Methods

Enzyme-linked immunoabsorbent assay (ELISA) was performed in the presence or absence of different enzymatic and specific receptors’ antagonist drugs. The levels and the generation of PGE2, 6-keto-PGF1α, and cyclic AMP (cAMP) in rat submandibular gland acini’s preparations and in serum from pSS patients were measured in the presence of pSS IgG anti M1 peptide. COX-2 mRNA gene’s expression at Real Time PCR was done in acini’s preparations from rat submandibular gland in the presence of the autoantibodies alone or once previously incubated with different inhibitors.

Results

In this study, we show that the activation of M1 mAChR of rat submandibular gland acini’s preparation triggers an increment both in the production of COX-2 mRNA gene’s expression and in the production of PGE2, 6-keto-PGF1α. These phenomena are accompanied by an increment in the production of cAMP in the acini’s preparation and do not affect COX-1 mRNA’s levels. Both prostanoids are augmented in the sera of pSS patients as compared with healthy individuals.

Conclusions

The present study suggests a complex interplay between different factors involved in adaptativa autoimmunity in pSS patients at the level of exocrine glands. The presence of pSS IgG anti M1 peptide, the enhancement of COX-2 mRNA gene’s expression and the increment in the generation of PGE2, and 6-keto-PGF1α abolished by M1 specific cholinergic antagonist, could provide evidence of a link between autoimmunity and the submandibular gland parasympathetic system in the course of Sjögren’s syndrome. This evidence is further supported by an increment in the production of AMP cyclic nucleotide (cAMP), and the subsequent induction of desensitization, internalization and/or intracellular degradation of the glandular M1 mAChR displayed by the cholinergic autoantibody. All of these statements cited above, are responsible for xerostomy, xerophthalmia and other parasympathetic symptoms observed in SS patients.

Keywords: Autoantibodies; Sjögren syndrome; PGE2; PGI2; cAMP; COX-2 mRNA

Introduction

Primary Sjögren’s syndrome (pSS) is a common chronic autoimmune disease characterized by the inflammation of exocrine glands and the clinical signs of xerostomia and keratoconjunctivitis sicca. A combination of environmental, genetic and possibly hormonal factors leads to the dysregulation of the glandular epithelium, mononuclear cell infiltration and abnormal lymphocyte activation and proliferation [1,2]. Alter humoral autoimmune responses, B cell hyperactivity and autoantibodies production are the hallmarks of pSS [3-5].

We have reported the presence of autoantibodies against rat salivary and lacrimal glands M1 muscarinic acetylcholine receptors (mAChR M1), which trigger parasympathetic-receptor-mediated biological effects [6-9]. We also demonstrated that they are able to recognize a synthetic peptide corresponding in amino acid sequence to the second extracellular loop of the human M1 mAChR. An isolated fraction of pSS IgG rich in anti M1 peptide antibodies could reproduce the effects of the corresponding whole immunoglobulin.

These facts suggest a prominent role of the anti M1 peptide antibody in the mAChR-mediated effects of total pSS IgG.

Other known facts are that the synthetic M1 peptide involved selectively suppresses the biological effects of SS anti M1 peptide autoantibody and the corresponding total IgG. The neurotransmitter mAChR autoantibodies coexist with the presence of other important autoantibodies to Ro/SS-A.
Preparation of submandibular gland acini's

Submandibular gland acini's were prepared from adult female Wistar-strain rats. Animals were used according to "The Guide to the Care and Use of Experimental Animals" (DHEW Publication, NIH 80-23). Glands were dissected away from fat, connective tissue and lymph nodes and immersed in a tissue chamber containing Krebs-Ringer-bicarbonate (KRB) solution gassed with 5% CO$_2$ in oxygen and maintained at pH 7.4 and 37°C. All subsequent steps were performed at 4°C. Submandibular glands were minced and incubated in KRB supplemented with 10 mM HEPES and 5.5 mM glucose (KRB-HEPES) and 0.5% bovine serum albumin (BSA), pH 7.4, containing collagenase (150 U/ml). Gland lobules were subjected to gentle pipetting. The preparation was then filtered through nylon mesh (150 μm pore size) and the acini's were pellets with a 2 min centrifugation at 50 g. The pellet was then washed twice by centrifugation (50 g for 2 min) through a 4% BSA solution made with KRB-HEPES buffer. The dispersed acini's were allowed to recover for 30 min in 5 ml fresh KRB-HEPES buffer containing 0.5% BSA [19].

Patients

The subjects of this study were 28 pSS anti-Ro/SSA positive patients and 25 healthy volunteers, all female, aged 39–54 years. They were selected from the metropolitan area of Buenos Aires (Table 1). The diagnosis of pSS fulfilled the criteria described by Vitali et al. [20] and was given by a panel of experts based on diagnostic criteria. All patients were female and had no evidence of systemic disease.

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 [21]. 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.

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 dilution 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 (60 kD and 52 kD 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 25 U/ml was considered positive (Table 1).

Anti-M, peptide IgG procedure: The IgG fraction from 28 patients with pSS and 25 healthy subjects was independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described by Reina et al. [8]. 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 antimuscarinic receptor peptide antibodies was determined by a radial immunodiffusion assay. Their immunological reactivity against muscarinic receptor peptides was evaluated by using ELISA. The concentration of the affinity-purified anti-M, peptide IgG (1×10$^5$ M) increased optical density (mean OD ± SEM; 2.4 ± 0.2). The non-affinity-purified, peptide IgG fraction eluted 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

(RO52, RO60) or La/SS-B [10-12]. These autoantibodies are non-organ specific. Their role in the pathogenesis of SS has not been understood yet.

This supports the view that the second extracellular loop is not only the main immunogenic region of the receptor but can be considered essential for the biological action of these autoantibodies.

In this context, it is important to notice that antinuclear antibodies (ANA) are frequently found together with the rheumatoid factor (RF) and anti centromere antibody (ACA) in patients with pSS in the early stages of the disease and at a younger age. These autoantibodies are not specific to SS, but show a local response to autoantigens derived from salivary glands aggressors, which produce these autoantibodies at the level of the local system [13]. Prostaglandins (PGs) are implicated in normal cellular processes as well as in pathophysiological conditions such as inflammation [14,15]. PGE, and PGI2 are synthesised by cyclooxygenase (COX) and prostaglandin and prostacyclin E and I synthesise in vivo. The two enzymes catalyse the reaction of transformation of arachidonic acid (AA) through PGH2 into PGE and PGI2. The two isoforms of COX (COX-1 and COX-2) and PGE, / PG12 have been identified: In general, COX-1 is constitutively expressed in almost all tissues and have haemostatic effects, whereas inflammatory processes lead to an increased expression of COX-2 [16] in response to inflammation; the increased expression of COX-2 provokes, in turn, an increment in the generation of PGE2 and PGI2; PGE, has been shown to be part of the signalling events involved in M$_3$ mAChR activation [17,18].

Accordingly, the aim of our work is to investigate if the inflammatory process provoked by the M$_3$ mAChR IgG of sera of pSS patients in rat submandibular gland and the following increased expression of mRNA COX-2 and production of PGE, and PGI2 are associated with the presence of M$_3$ mAChR IgG.

Material and Methods

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.

Drugs

A 25-mer peptide (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) corresponding to the sequence of the second extracellular loop of the human M$_3$ mAChR was synthesized by Peptide Genetic Research Company (Livermore, CA, USA) as previously described [6]. DuPe697 5 × 10$^{-5}$ M (5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl-thiophene); RO3144794 5 × 10$^{-4}$ M (R-3-(4-fluoro-phenyl)-2-[3-(4-fluoro-phenyl)-benzofuran-2-ylmethoxycarbonylamino]-propionic acid); PF-04418948 2 × 10$^{-3}$ M (1-(4-fluorobenzoyl)-3-((6-methoxy-2-naphthyl)oxy) [methyl] azetidine-3-carboxylic acid) were from Tocris Cookson (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers and the drugs were diluted in water to achieve the final concentrations stated in the text.

Animals

Male Wistar rats weighing 250-300 g from the Pharmacologic Bioterium School of Dentistry, University of Buenos Aires) were used throughout. The animals housed in standard environmental conditions were fed with a commercial pellet diet and water ad libitum. For surgical removal of submandibular glands, the animals were killed with an overdose of an i.p. ketamine/xylazine mixture (100 and 16 mg/kg respectively). The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

PGE₂ were 42.6 ng/L and 6-keto-PGF₁α 64.2 ng/L; the OD cut-off values for gland acini’s PGE₂ were 0.5 ng/ml and 6-keto-PGF₁α 0.62 ng/ml (basal control). All gland acini’s samples were frozen promptly after collection and kept at -80°C until used for PGE₂ and 6-keto-PGF₁α determination. The result in serum is expressed as ng/ml and in submandibular gland acini's in ng/ml.

### Determination of cyclic AMP levels

To determine cyclic AMP (cAMP) levels tissues (submandibular gland acini) were incubated in 1 ml of Krebs Ringer Bicarbonate (KRB) for 30 min. After incubation, submandibular gland acini were homogenized in 2 ml of absolute ethanol and centrifuged at 6000 g for 15 min at 4°C. Pellets were then re-homogenized in ethanol–water (2:1) and supernatants were collected and evaporated to dryness. The residue was dissolved in 400 μl of 50 μM sodium acetate buffer, pH 6.2 and aliquots of 100 μl were taken for nucleotide determination using a radio immunoassay procedure with a 3H-cAMP-RIA kit from Dupont New England Nuclear (Boston, MA, USA).

### Statistical analyses

The Student’s “t” test for unpaired values was used to determine the level of significance. In case multiple comparisons were necessary after analysis of variance the Student-Newman-Keuls test was applied. Differences between means were considered significant if P<0.05.

### Results

#### Effect of pSS IgG anti M₁ peptide

To determine the effect of pSS IgG anti M₁ peptide on gland acini’s we analyzed the time-course of COX-2 mRNA expression by Real Time-PCR (Figures 1A and B). At the sixth hour presence of pSS IgG anti M₁ (1 × 10⁻⁶ M), COX-2-mRNA was significantly (P<0.001) increased versus normal IgG anti M₁, which is ineffective in this studied system (control). When the autoantibody was incubated in the presence of DuP697 (1 × 10⁻⁶ M) [a specific COX-2 antagonist] under the same experimental conditions, it abrogated the increment of COX-mRNAs expression in a significant manner (P<0.01). However, when the experimental protocol was assessed in the presence of M₁, mACHr specific antagonist J104127 (1 × 10⁻⁴ M) and/or synthetic M₁ peptide (5 × 10⁻⁶ M) the change in COX-2 mRNA expression by the pSS IgG anti M₁ did not reach statistical significance (Figure 1C). COX-1 mRNA expression did not change significantly in our preparation system.

#### Effect of pSS IgG anti M₁ on the production of PGE₂ and 6-keto-PGF₁α

As shown in Figure 2, the dose-response concentration curve of PGE₂ (A) and 6-keto-PGF₁α (C) in the presence of pSS IgG anti M₁ on submandibular gland acini’s increased the production of both prostanoids, reaching the maximal action when the antibody is at a concentration of 1 × 10⁻⁴ M. The increment in the generation of both prostanoids is abrogated, reaching values similar to basal ones, when the tissue preparations are incubated with prostaglandin antagonists PF-04418948 2 × 10⁻⁶ M (Figure 2A) and RO3244794 5 × 10⁻⁶ M (Figure 2C) for PGE₂, and 6-keto-PGF₁α respectively, and in the presence of synthetic M₁ peptide 5 × 10⁻⁶ M (Figures 2A and 2C). Normal (n) IgG anti M₁ peptide was ineffective in our study system (Figure 2 histogram B and D).

#### Effect of pSS IgG anti M₁ on the production of cyclic AMP

Synthetic M₁ peptide 5 × 10⁻⁶ M, PGE₂ 5 × 10⁻⁶ M and 6-keto-PGF₁α 5 × 10⁻⁶ M were accompanied by modification of cAMP production associated positively with the increased amount of both prostanoids (Figure 3 and Table 2) and parenthesis (data not shown).
In Figure 3A it can be seen that the production of cAMP reaches maximal increment at $1 \times 10^{-8} \text{M}$ pSS IgG anti M3 peptide. This stimulatory effect was almost abolished by pre-treating submandibular gland acini's with J104127 $1 \times 10^{-8} \text{M}$ and/or synthetic M3 peptide $5 \times 10^{-6} \text{M}$. The normal IgG anti M3 peptide gave negative results (Figure 3A).

To ascertain if this cAMP increment is responsible for the augmented generation of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ in our preparation, we study the action of both prostanoids on the production of this nucleotide. Figure 3B and Figure 3C show that both prostaglandins are able to increase cAMP production, whereas the selective prostanoids antagonists (PF-04418948 for PGE$_2$ and RO3244794 for 6-keto-PGF$_{1\alpha}$) blunt the stimulatory action provoked by the prostaglandins.

**Discussion**

In this study, we show that the activation of M$_3$ mAChR of rat submandibular gland acini's preparation triggers an increment in the production of COX-2 mRNA gene's expression together with an increment in the production of PGE$_2$ and 6-keto-PGF$_{1\alpha}$. These phenomena are accompanied by an increment in the production of cAMP in acini's preparation without affecting COX-1 mRNA levels.

Positive regulation by cholinergic agonists has been described in different tissues [24-27] including rat submandibular gland [28]. We observed that such stimulation was due to COX-2's activation through M$_3$ mAChR glandular acini's activation: this increment was prevented by the specific blockade of this enzyme [29], by the cholinoceptor antagonist agent [30] and by M$_3$ synthetic peptide [16]. However, the rat submandibular gland constitutively expressed COX-1 [31]. In most cells, COX-1 mediates

Figure 1: Bar graph depicts the mRNA expression of cyclooxygenase-2 (COX-2) in rat submandibular gland acini's. The acini's from eight rats were treated during 3 hours with or without (basal) pSSIgG (1x10^{-7} \text{M}) or IgG anti M$_3$ peptide (1x10^{-6} \text{M}) or n IgG anti M$_3$ IgG peptide from healthy individuals (normal: n IgG) and primary Sjögren's syndrome patients (pSS patients) respectively. In inhibitory experiments, COX-2 inhibitor (DuP697 $5 \times 10^{-6} \text{M}$), M$_3$ specific mAChR antagonist (J104127 $1 \times 10^{-8} \text{M}$ and M3 synthetic peptide (1x10^{-5} \text{M}) were added to acini's 30 minutes before pSS IgG or pSS IgG anti M3 treatment. The COX-2 mRNA was extracted using quantitative real-time polymerase chain reaction and the levels of COX-2 were measured. Values are mean ± SEM expressed as per cent of change of 5 experiments in each group performed by duplicate. *P<0.0001 versus basal (acini's alone); **P<0.001 versus pSS IgG or pSS IgG anti M$_3$.

Figure 2: A: Concentration-response curve of pSS IgG anti M$_3$ peptide alone (●) or in the presence of PF-04418948 2x10^{-9} \text{M} (▲) and J104127 1x10^{-8} \text{M} (▲) on the generation of PGE$_2$. B: Histogram shows in comparative form in acini's preparations alone, the action of the maximal concentration (1x10^{-8} \text{M}) of pSS IgG anti M$_3$ peptide in comparison to normal (n) IgG anti M$_3$ peptide on the generation of PGE$_2$. C: Concentration-response curve of pSS IgG anti M$_3$ peptide alone (●) or in the presence of RO3244794 5x10^{-8} \text{M} (▲) and J104127 1x10^{-8} \text{M} (▲) on the generation of 6-keto-PGF$_{1\alpha}$. D: Histogram shows in comparative form in acini's preparations alone, the action of the maximal concentration (1x10^{-8} \text{M}) of pSS IgG anti M$_3$ peptide in ○Values are mean ± SEM of 5 experiments in each group performed by duplicate; *P<0.001 versus basal and nIgG anti M3 values.

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physiological responses such as the modulation of the autonomic nervous system, whereas, COX-2 mainly plays a role in inflammation, infection and cellular proliferation [22]. In our case, the autoantibody from sera of pSS patients was able to stimulate the COX-2 mRNAs expression. This was the case due to the fact that when this pSS IgG anti M₃ peptide binds to, and activates, the M₃ mAChR receptor present in the membrane of gland acinar cells; it provokes an inflammatory process, in which COX-2 is expressed [23]. COX-1’s expression did not change significantly in the rat submandibular gland acini’s preparations in the presence of the cholinergic autoantibody.

On the other hand, pSS IgG anti M₃ peptide has a capacity to stimulate the production of PGE₂ and 6-keto-PGF₁α, in concentration-response curve fashion in gland acini’s preparations. However, the stimulatory action of these autoantibodies is inhibited by M₃ mAChR’s specific antagonist [30], by PGE₂’s specific antagonist [31], 6-keto-PGF₁α’s antagonist [32] and M₃ synthetic peptide [16]. This shows that the action of these autoantibodies on acini’s glandular preparations act as an inducer of the augmented generation of both prostanoids. The prostanoids, in turn, are able to contribute to the vasodilatory blood flow in all tissues acting as proinflammatory cytokine [35]. Both prostanoids can be considered to play a crucial role in the process of inflammation (occurring at the level of the exocrine glands, i.e., eye and salivary gland), which is itself responsible for the xerophthalmia and xerostomia present in pSS provoked by the cholinergic autoantibody.

In all these pharmacological approaches the increment of COX-2 / PGE2 / PG12 in the submandibular gland acini’s preparations through the action of pSS IgG anti M₃ peptide is shown to be the result of the ability of this cholinergic autoantibody present in the sera of pSS patients to mobilize arachidonic acid from phospholipids. The presence of this autoantibody is shown to lead to the subsequent activation and increase of the COX-2 mRNAs expression accompanied by a large amount of PGE₂ / PG12. Both prostanoid are thus shown to be responsible of the installation of the neuroinflammatory pathophysiological processes at the level of glandular salivary gland.

The increment in PGE₂ and 6-keto-PGF₁α is mediated by cAMP’s increased production, whereas the increased production of this nucleotide...
is inhibited by the prostanoids' specific antagonists. In acini's preparations pSS IgG anti M₁ peptide is able per se to enhance cAMP's production and 1α, P<0.0001.

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Conflict of Interest
The authors declare no conflict of interest.

References


