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Anti-M_3 muscarinic cholinergic autoantibodies from patients with primary Sjögren's syndrome trigger production of matrix metalloproteinase-3 (MMP-3) and prostaglandin E_2 (PGE_2) from the submandibular glands

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ABSTRACT

Background: We demonstrated that serum immunoglobulin G (IgG) from patients with primary Sjögren's syndrome (pSS), interacting with the second extracellular loop of human glandular M_3 muscarinic acetylcholine receptors (M_3 mAChR), trigger the production of matrix metalloproteinase-3 (MMP-3) and prostaglandin E_2 (PGE_2).
Methods: Enzyme-linked immunosorbent assays (ELISAs) were performed in the presence of M_3 mAChR synthetic peptide as antigen to detect in serum the autoantibodies. Further, MMP-3 and PGE_2 production were determined in the presence of anti-M_3 mAChR autoantibodies.
Results: An association was observed between serum and anti-M_3 mAChR autoantibodies and serum levels of MMP-3 and PGE_2 in pSS patients. Thus, we established that serum anti-M_3 mAChR autoantibodies, MMP-3 and PGE_2 may be considered to be early markers of pSS associated with inflammation. Affinity-purified anti-M_3 mAChR peptide IgG from pSS patients, whilst stimulating salivary-gland M_3 mAChR, causes an increase in the level of MMP-3 and PGE_2 as a result of the activation of phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2) (but not COX-1).
Conclusions: These results provide a novel insight into the role that cholinoceptor antibodies play in the development of glandular inflammation. This is the first report showing that an antibody interacting with glandular mAChR can induce the production of pro-inflammatory mediators (MMP-3/PGE_2).

1. Introduction

Sjögren's syndrome is considered to be an auto-immune disease characterised by chronic inflammation involving the salivary glands. Sjögren's syndrome also reveals the complex interaction between innate and adaptive immunity and dysfunction of the salivary glands.

Autoreactive lymphocytes[1,2] and antibodies against M_3 muscarinic acetylcholine receptors (mAChRs)[3] have been described in Sjögren's syndrome. Our research has been based on the hypothesis that antibody–cell interactions, mediated through mAChR in the salivary glands, determine dysfunction in the salivary glands by triggering molecular alterations and inexorably lead to damage to exocrine glands.[4–6]

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Fixation of M_3 mAChR autoantibodies from patients with primary Sjögren's syndrome (pSS M_3 immunoglobulin G (IgG)) has functional implications for the salivary glands. This is because this limits not only parasympathetic stimulation with decreased salivary function, but also the effectiveness of endogenous agonists.[7] It is well known that the primary effector mechanism activated by M_3 mAChR is generation of the secondary messengers inositol phosphate ($InsP_3$) and prostaglandin E_2 (PGE_2). Each of these metabolites not only influences salivary secretion by mobilising calcium from intracellular stores and regulating the absorption of ions and water,[8] but also plays a key role in the pathophysiology of chronic inflammation.[9] These facts have led us to think that the damage and inflammation in the salivary glands commonly seen in pSS patients might be a consequence of the production of pro-inflammatory mediators induced by antibody–mAChR interaction on gland membranes.

In the salivary glands, the basal lamina of the acini is connected to the cytoskeleton of acinar cells via integrins in the basal plasma membrane.[10,11] Matrix metalloproteinase-3 (MMP-3) degrades components of the basal lamina,[11] and might be involved in loosening of cell anchorage to the basal lamina. As a consequence of such changes, inhibition of the proliferation, differentiation and regeneration of epithelial cells,[12] as well as activation of apoptosis,[13,14] may account for salivary-gland damage. Moreover, patients with pSS show elevated levels of MMP-3 in their saliva.[15]

Prostaglandins (PGs) have been implicated in normal cellular processes as well as in pathophysiological conditions such as inflammation.[16] Nitric oxide (NO) plays a key part in the pathophysiology of chronic inflammation and in the neurodegenerative process.[17] PGE_2 is synthesised by cyclooxygenase (COX) and prostaglandin E synthase (PGES) *in vivo*; the two enzymes catalyse the reaction of transformation of arachidonic acid (AA) through PGH_2 into PGE_2. The two isoforms of COX (COX-1 and COX-2) and PGEs (cytosolic (cPGEs) and membrane (mPGEs)) have been identified. In general, COX-1 and cPGEs are constitutively expressed in almost all tissues and have haemostatic effects, whereas COX-2 and mPGEs are inducible enzymes that are expressed in response to inflammation.[18] PGE_2 has been shown to be part of the signalling events involved in M_3 mAChR activation.[19,20]

We hypothesised that, in addition to direct alteration of salivary-gland function, M_3 mAChR autoantibodies may contribute to the inflammatory reaction in the salivary glands observed in chronic pSS.[19]

We investigated whether antibodies against M_3 mAChR can induce cholinergic MMP-3 activation and PGE_2 generation. If so, this would provide novel insights into how anti-M_3 mAChR antibodies activate phospholipase A_2 (PLA_2) and COX-2 to trigger the production of pro-inflammatory mediators. The present study provided evidence that anti-M_3 mAChR antibodies and PGE_2 in serum may serve as early markers of pSS.

2. Materials and methods

2.1. Study population

Females (age: 35–55 years) were selected from the metropolitan area of Buenos Aires. Subjects were divided into two groups: 17 patients with pSS and 15 healthy volunteers. The diagnosis of pSS followed the four or more criteria stated by Vitali et al.[21] All 17 patients (100%) gave a positive biopsy with a score focus of 3.8 ± 0.07. Serologic tests were carried out for anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF) and antinuclear antibodies (ANA) (Table 1). The study 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 provided written informed consent.

Table 1 – Serological tests carried out on pSS patients and normal individuals.

Serological test	pSS patients	Healthy individuals
ANA	13/17 (76%)	1/15 (6%)
Anti-Ro (SS-A)	8/17 (47%)	0/15 (0%)
Anti-La (SS-B)	7/17 (41%)	0/15 (0%)
RF	6/17 (35%)	1/15 (6%)

2.2. M_3 mAChR synthetic peptide

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 amino-acid sequence of the second extracellular loop of the human M_3 mAChR was synthesised by F-moc-amino acids activated using a 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBt/DCC) strategy with an automatic peptide synthesiser (Model 431A, Applied Biosystems; Menlo Park Ct, CA, USA). The peptide was desalted and purified by high-performance liquid chromatography (HPLC). It was then subjected to amino-terminal sequence analysis by automatic Edman degradation (470 A Sequence, Applied Biosystems).

2.3. Purification of human IgG

The serum IgG fraction from 17 patients with pSS and 15 normal subjects was isolated by protein G-affinity chromatography for protein A and standardised for protein G. Briefly, sera were loaded onto the protein G (Sigma, Saint Louis, MO, USA)-affinity column equilibrated with 1 M Tris–HCl, pH = 8.0. The columns were then washed with 10 vol of the same buffer. The IgG fraction was eluted with 100 mM glycine–HCl (pH = 3.0) and immediately neutralised. IgG concentrations were determined by a radial immunodifusion assay.

2.4. Purification of anti-M_3 peptide IgG by affinity chromatography

The IgG fractions obtained from 17 patients with pSS and 15 normal subjects were independently subjected to affinity chromatography on the synthesised peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described.[3] Briefly, the IgG fraction was loaded onto the affinity column equilibrated with phosphate-buffered saline (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 were determined by a radial immunodiffusion assay. Their

immunological reactivity against muscarinic receptor peptides was evaluated by enzyme-linked immunosorbent assay (ELISA). The concentration of the affinity-purified anti-M_3 peptide IgG (1×10^{-7} M) that maximally increased optical density (OD, 2.4 ± 0.2) corresponded to a total IgG concentration of 1×10^{-6} M (OD, 2.2 ± 0.2). The non-anti-M_3 peptide IgG fraction eluted from the column showed OD values (0.27 ± 0.06) similar to those of normal IgG (OD, 0.25 ± 0.04). The normal IgG fraction purified by affinity column chromatography gave a negative result (OD, 0.24 ± 0.03).

2.5. ELISA

Fifty microlitres of M_3 mAChR peptide solution in 0.1 M Na_2CO_3 buffer (pH = 9.6) was used to coat microtitre plates (COSTAR) at 4 °C overnight as decribed.[3] After blocking the wells, different dilutions of purified IgG from patients with pSS and normal subjects were allowed to react with the antigens for 2 h at 37 °C. The wells were then thoroughly washed with Tween 20 in PBS. Goat anti-human IgG avidin–alkaline phosphatase (50 μl) was added and incubated for 1 h at 37 °C. After several washing steps, p-nitrophenyl phosphate (1 mg ml^{-1}) was added as the substrate; the reaction was stopped at 30 min. OD values were measured using an ELISA reader (Uniskan Laboratory System, Helsinki, Finland). As negative controls, non-antigen paired wells and wells with no primary antiserum were also tested.

2.6. PGE_2 and MMP-3 assays

Slices of submandibular glands were incubated for 60 min in 0.50 ml Krebs Ringer bicarbonate (KRB) gassed with 5% CO_2 in oxygen at 37 °C. IgG or pilocarpine was added 30 min before the end of the incubation period. Blockers were added 30 min before the addition of different concentrations of IgG or pilocarpine. Glands were then homogenised into a 1.5-ml polypropylene microcentrifuge tube. Thereafter, all procedures employed were those indicated in the protocol of Prostaglandin E_2 Biotrak Enzyme Immunoassay (ELISA) System (Amersham Biosciences, Piscataway, NJ, USA). For extraction of extracellular matrix components, the methods described by Rapraeger et al.[22] were used with minor modifications. Briefly, samples were mixed with extraction buffer (0.5 M Tris–HCl (pH 7.5), 1% Triton X-100, 10 mM $CaCl_2$, 200 mM NaCl) in a ratio of 1:5 (weight/volume) at 4 °C and homogenised in a glass/glass conical homogeniser. The homogenate was then subject to three freeze–thaw cycles of 5 min each and centrifuged at 13,000 × *g* for 30 min at 4 °C. The detergent-soluble supernatant was recovered and stored at −70 °C for further analysis, and the insoluble pellet fractions discarded. Protein determination was according to the method of Lowry and Thiessen.[23] Detergent extracts of glands were analysed by ELISA to determine the enzymatic activity of MMP-3 according to the manufacturer's instructions (Amersham Matrix Metalloproteinase-3, Biotrak Activity Assay System, GE Healthcare, NJ, USA). PGE_2 and MMP-3 results were expressed as ng ml^{-1} and μg ml^{-1}, respectively.

2.7. Drugs

Pilocarpine and 4-diphenyl-acetoxy-*N*-methyl-piperidine methiodide (4-DAMP) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA); 4-(4-octadecylphenyl)-4-oxobutenoic acid (OBAA), 1-[4,5-bis(4-methoxyphenyl)-2-thiazolyl] carbonyl-4-methylpiperazine hydrochloride (FR-122047), (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulphonyl)phenyl]-thiophene) (DuP 697) and cyclopenthyl-α-hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl] benzeneacetamide fumarate (J 104129) were purchased from Tocris Cookson Incorporated (Baldwin, MO, USA). Stock solutions were freshly prepared in the corresponding buffers. Drugs were diluted in a waterbath to achieve that final concentration stated in the text.

2.8. Statistical analyses

Student's *t*-test for unpaired values was used to determine the level of significance. If multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $p < 0.05$.

3. Results

3.1. Detection of M_3 mAChR and the production of MMP-3 and PGE_2 in serum

ELISA assays were carried out to ascertain if there was a correlation between serum IgG and M_3 mAChR synthetic peptide (Fig. 1(A)), serum MMP-3 (Fig. 1(B)) as well as serum PGE_2 levels (Fig. 1(C)) in pSS patients and normal subjects (control). Fig. 1 shows the OD values for serum from pSS patients or normal subjects. The OD values obtained from pSS sera were >2 SD than those from normal individuals. A positive correlation (alpha = 0.05) was observed between serum anti-M_3 peptide IgG titres and serum MMP-3 levels (Fig. 2(A)) and serum anti M_3 peptide IgG titres and PGE_2 levels (Fig. 2(B)) from pSS patients. Hence, sera that reacted positively against M_3 synthetic peptide showed high levels of MMP-3 and PGE_2.

3.2. Stimulation of generation of MMP-3 and PGE_2 in submandibular glands

Anti-M_3 peptide from pSS patients stimulated production of MMP-3 in a dose-dependent manner, with the maximal effect being at 1×10^{-8} M (Fig. 3(A)). The corresponding non-anti-M_3 peptide IgG gave negative results. The maximal effect of anti-M_3 peptide IgG on MMP-3 production was significantly higher than the maximal effect of pilocarpine (1×10^{-7} M) (Fig. 3(B)). The actions of anti-M_3 peptide IgG and pilocarpine were inhibited by J 104129 (4.5×10^{-9} M) and 4-DAMP (0.2×10^{-9} M); concentrations that correspond to the K_i of the respective M_3 mAChR antagonists.[24,25] As the control, the IgG fraction from normal individuals purified by affinity chromatography with M_3 mAChR synthetic peptide gave negative results.

The pSS anti-M_3 mAChR peptide IgG could also stimulate the production of PGE_2 in slices of submandibular glands (Fig. 4(A)). The effect was dependent upon IgG concentration. The corresponding IgG fraction eluted from the column with M_3 mAChR synthetic peptide (non-peptide fraction) gave negative results. The effect of M_3 mAChR peptide IgG resembled that of

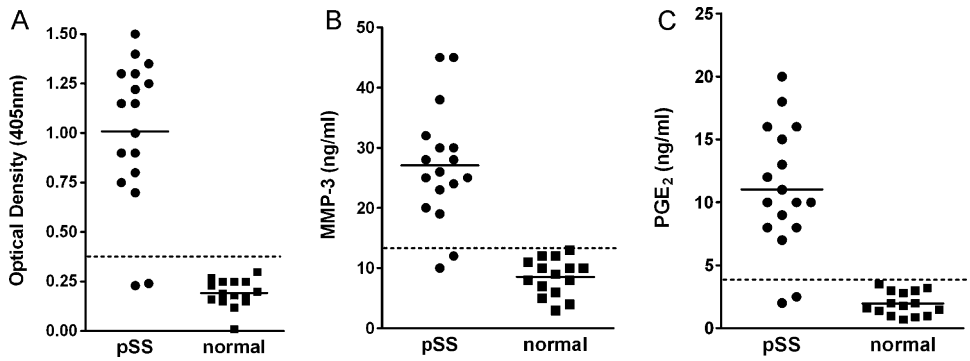
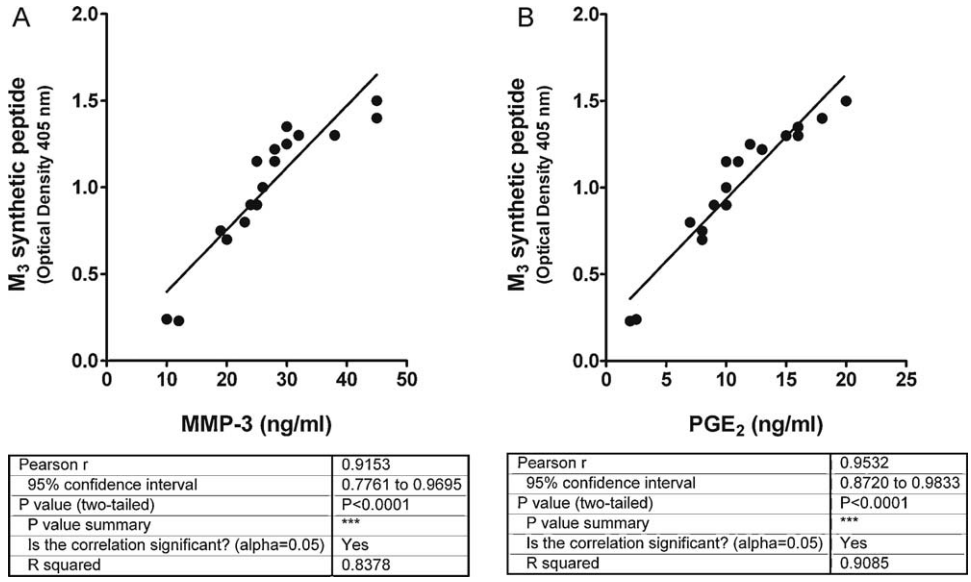


Fig. 1 – Detection of serum antibody titres and levels of MMP-3 and PGE_2. Scattergram showing the immunoreactivity of circulating IgG antibodies against M_3 mAChR synthetic peptide (A), serum MMP-3 (B) and serum PGE_2 (C). Individual optical density (OD) values for each serum (1/30 dilution) from 17 pSS patients and 15 healthy individuals (normal). OD cutoff values of 0.30 ± 0.01,12.6 ± 0.11, and 4.4 ± 0.33 for anti-M_3 mAChR IgG, serum MMP-3, and serum PGE_2 respectively.



Pearson r	0.9153
95% confidence interval	0.7761 to 0.9695
P value (two-tailed)	P<0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.8378

Pearson r	0.9532
95% confidence interval	0.8720 to 0.9833
P value (two-tailed)	P<0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.9085

Fig. 2 – Correlation between serum antibody titres and levels of MMP-3 and PGE_2. Correlation between titres of serum anti-M_3 mAChR peptide IgG and serum levels of MMP-3 (A) and PGE_2 (B) in pSS patients. Anti-M_3 mAChR peptide IgG (pSS IgG) titres were plotted as a function of serum MMP-3 (A) or serum PGE_2 (B). Values correspond to 17 pSS patients.

pilocarpine. However, the maximal increment induced by 1×10^{-8} M autoantibodies was significantly higher than that of an authentic cholinergic agonist at 1×10^{-7} M (Fig. 4(B)). J 104129 (4.5×10^{-9} M) and 4-DAMP (0.2×10^{-9} M) decreased the stimulatory action of pSS IgG and pilocarpine on PGE_2 generation. Normal IgG was ineffective in this system.

Under identical experimental conditions, there was a significant correlation between anti-M_3 mAChR peptide IgG-stimulated production of PGE_2 and MMP-3 (Fig. 5). These results indicated that the increased production of MMP-3 due to activation of M_3 mAChR by anti-M_3 mAChR peptide IgG might be a result of stimulation of PGE_2 generation.

3.3. *Arachidonic acid (AA) cascade enzymes implicated in generation of MMP-3 and PGE_2 by anti-M_3 mAChR IgG*

To discern which arachidonic acid (AA) cascade enzymes are implicated in anti-M_3 mAChR peptide IgG-generated production of MMP-3 (Fig. 6(A)) and PGE_2 (Fig. 6(B)), several inhibitors of this enzymatic cascade were used. Inhibition of PLA_2 by OBAA (5×10^{-6} M) or COX-2 by DuP 697 (5×10^{-8} M) demonstrated the stimulatory action of anti-M_3 mAChR peptide IgG-induced production of MMP-3 (Fig. 6(A)) and PGE_2 (Fig. 6(B)). COX-1 inhibition by FR-122047 (5×10^{-8} M) had no effect on MMP-3 or PGE_2. These results indicated that PGE_2 mediates the anti-M_3 mAChR peptide IgG effect on MMP-3 production via PLA_2 and COX-2 activation, without the participation of COX-1.

4. Discussion

Sera from pSS patients contain high amounts of MMP-3 and PGE_2 that correlates with the presence of serum anti-M_3 mAChR autoantibodies. These autoantibodies recognise the submandibular gland membrane and can interact with the second extracellular loop of human M_3 mAChR. The molecular

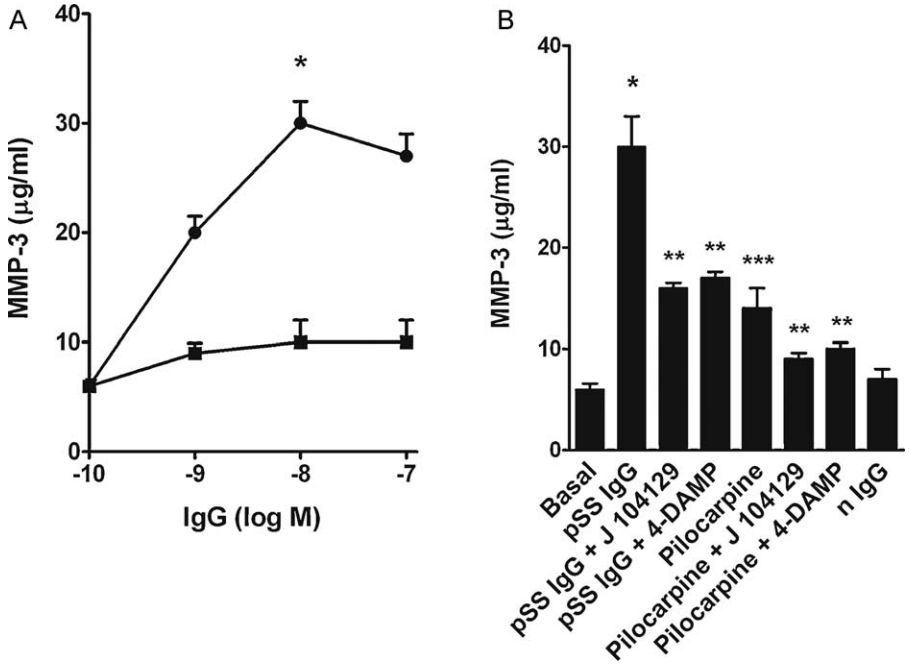


Fig. 3 – Stimulation of glandular MMP-3 production by pSS IgG. (A) Dose–response curve of anti-M_3 mAChR peptide IgG (pSS IgG) from pSS patients (●) and pSS IgG eluted from M_3 synthetic peptide (non-anti-M_3 peptide IgG) (■) induced stimulation of MMP-3 production by salivary glands. (B) Bar plot: 1×10^{-8} M anti-M_3 mAChR peptide IgG (pSS IgG) and 1×10^{-7} M pilocarpine (maximal effect) on MMP-3 production in absence or presence of J 104129 (4.5×10^{-9} M) and 4-DAMP (0.2×10^{-9} M). As control, the effect of 1×10^{-7} M normal IgG (nIgG) is also shown. Values are mean ± SEM of 17 pSS IgG, 15 normal IgG, or 6 pilocarpine experiments in each group. *P < 0.001 versus basal; **P < 0.001 versus pSS IgG and pilocarpine; ***P < 0.001 versus pSS IgG alone.

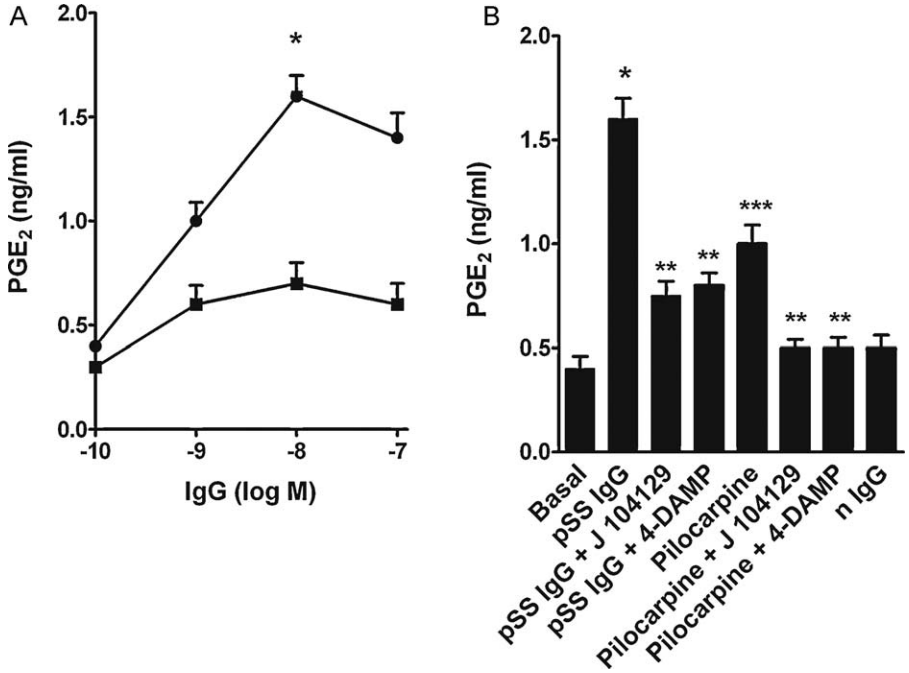
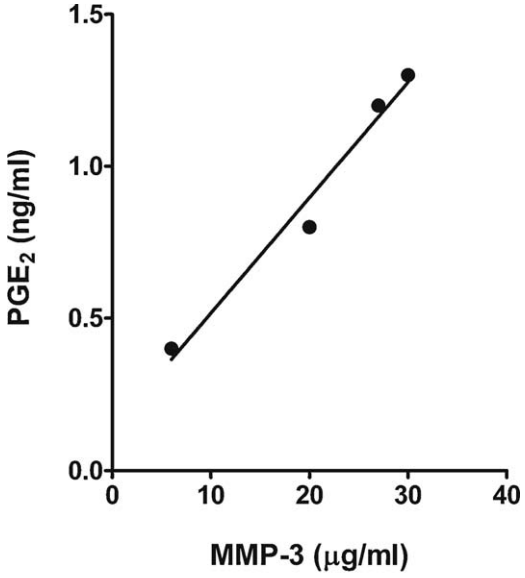


Fig. 4 – Stimulation of glandular production of PGE_2 by pSS IgG. (A) Dose–response curve of anti-M_3 mAChR peptide IgG from the same pSS patients (pSS IgG, ●) and non-anti-M_3 peptide IgG from healthy subjects (normal, ■). (B) Bar plot: anti-M_3 mAChR peptide IgG (pSS IgG) (1×10^{-8} M) and 1×10^{-7} M pilocarpine (maximal effects) on PGE_2 production in the absence or presence of J 104129 (4.5×10^{-9} M) and 4-DAMP (0.2×10^{-9} M). As control, the effect of 1×10^{-7} M normal IgG (nIgG) is also shown. Values are mean ± SEM of 17 pSS IgG, 15 normal IgG, or 6 pilocarpine experiments in each group. *P < 0.001 versus basal; **P < 0.001 versus pSS IgG or pilocarpine alone; ***P < 0.001 versus pSS IgG alone.



Pearson r	0.9876
95% confidence interval	0.5206 to 0.9998
P value (two-tailed)	0.0124
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.9753

Fig. 5 – Correlation between glandular production of MMP-3 and PGE_2 triggered by pSS IgG. Correlation of the stimulatory effect of anti-M_3 mAChR peptide IgG from pSS patients on MMP-3 and PGE_2 production and the ratio of MMP-3/PGE_2 was plotted as a function of MMP-3.

interactions of pSS antibodies with glandular M_3 mAChR can generate the pro-inflammatory substances MMP-3 and PGE_2, correlating with glandular inflammation and possibly contributing to the pathogenesis of pSS.

We found an association between functionally active serum anti-M_3 mAChR peptide IgG with xerostomia[4] and xerophthalmia[5] in pSS patients. In the present study, the finding of a significant correlation between serum anti-M_3 mAChR antibody titres and serum levels of MMP-3 and PGE_2 in pSS patients suggests that these autoantibodies may serve as markers of glandular inflammation. Moreover, serum anti-M_3 mAChR IgG induced the production of MMP-3 and PGE_2 by the salivary glands. The similarities between these experiments and clinical studies pose a question: could there be a common explanation for the mechanism by which IgG triggers the inflammatory process and mediates glandular dysfunction?

We demonstrated that anti-M_3 mAChR peptide IgG, behaving as a cholinergic agonist, triggered the production of MMP-3 and PGE_2 in response to receptor-mediated signalling events at the cell membrane.

The increased production of MMP-3 and PGE_2 is dependent upon the activation of M_3 mAChR in the submandibular gland; these processes may also have some pharmacological support because they were blocked by J 104129 and 4-DAMP. Moreover, the fact that the affinity-purified IgG fraction (anti-M_3 peptide IgG), but not that eluted from the column (non-anti M_3 peptide IgG), was responsible of the biological effect, points to the specificity of the reaction. Analyses of the effects of PLA_2 inhibition on the increased production of MMP-3 and PGE_2 triggered by pSS IgG, as observed in the present study, suggest that the mechanism by which IgG up-regulated the expression of pro-inflammatory mediators involves stimulation of AA cascade pathways that subsequently activate COX-2. The

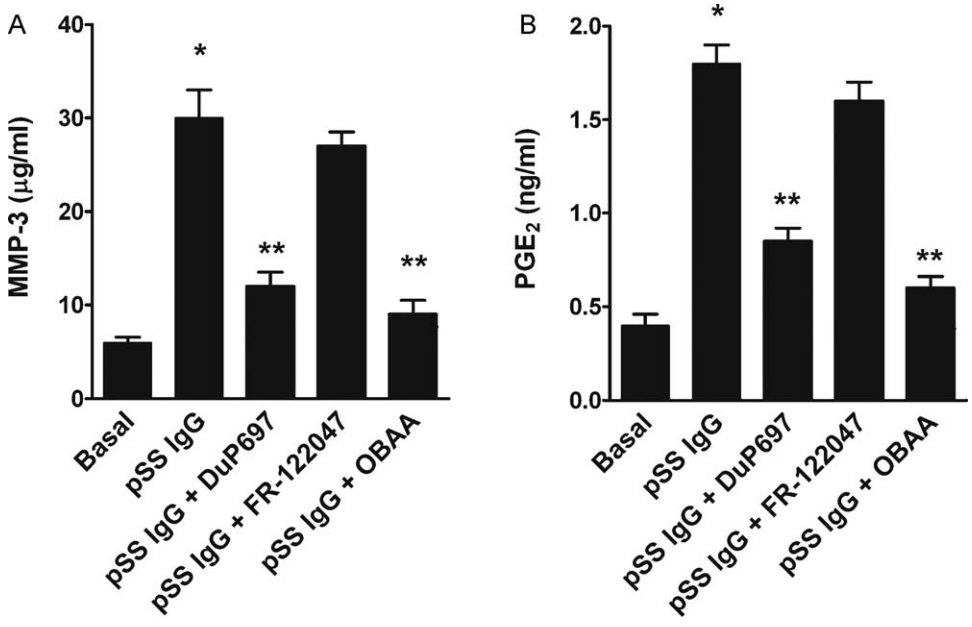


Fig. 6 – Arachidonic acid cascade enzymes implicated in pSS IgG-generated production of MMP-3 and PGE_2 by submandibular glands. Effect of anti-M_3 mAChR peptide IgG from pSS (pSS IgG, 1×10^{-8} M)-induced MMP-3 (A) and PGE_2 (B) production alone or in the presence of 5×10^{-8} M DuP 697, 5×10^{-6} M FR 122047 and 5×10^{-6} M OBAA. Values are mean ± SEM of 10 pSS IgG experiments in each group. $^{*}P < 0.001$ versus basal; $^{**}P < 0.001$ versus pSS IgG alone.

production of PGE_2 and MMP-3 by the autoantibody was mainly generated by COX-2 activation because it was prevented by the specific blockade of this enzyme. Further, proteolysis in the basal lamina (increase in MMP-3 level) of the acini and ducts of the salivary glands of patients with pSS is a critical element in maintaining normal tissue architecture. An imbalance in the expression and activity of MMP-3 may lead to severe destruction of the salivary glands.[26]

The fact that inhibition of PLA_2 and COX-2 could inhibit the formation of MMP-3/PGE_2 by anti-M_3 mAChR peptide IgG indicated that anti-inflammatory substances might exert a beneficial effect on the prevention of acute inflammatory processes in the submandibular gland in pSS patients. In accordance with the present study, Xue et al.[27] reported that aspirin inhibits MMP-9 mRNA expression and COX-2/mPGES-1 in macrophages from THP-1 cells.

The present study suggests a complex interplay between different factors involved in innate and adaptive immunity. The presence of anti-M_3 mAChR peptide IgG and the increased production of MMP-3 and PGE_2 could provide a link between auto-immunity and exocrine dysfunction in Sjögren's syndrome. Further, the early agonist-promoting activation of salivary gland M_3 mAChR initiated by autoantibodies binds to and persistently activates cholinoceptors, resulting in the production of large amounts of pro-inflammatory MMP-3 and PGE_2, contributing to inflammation. The agonist activity displayed by anti-M_3 mAChR peptide antibodies could subsequently induce desensitisation, internalisation and/or intracellular degradation of glandular M_3 mAChR. This would lead to a progressive reduction in the surface expression and activity of glandular M_3 mAChR, resulting in xerostomy, xerophthalmia and other general symptoms in pSS patients.

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Competing interests: None declared.

Ethical approval: The study 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 provided written informed consent.

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