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AUTOANTIBODIES AGAINST SUBMANDIBULAR GLAND MUSCARINIC CHOLINOCEPTOR SUBTYPES IN PRIMARY SJÖGREN SYNDROME

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Sjögren Syndrome (SS) is a chronic autoimmune disease characterized by parasympathetic exocrine gland dysfunction. Here, the involvement of submandibular gland muscarinic acetylcholine receptor (mAChR) M_4 subtype is proposed as an IgG target together with M_1 and M_3 mAChR subtypes. The Kd values were total membranes 0.20 ± 0.017 nM; acini membranes 0.33 ± 0.023 nM and duct membranes 0.22 ± 0.040 nM and Bmax values were total, 1038 ± 24, acini, 1359 ± 28 and ducts, 593 ± 30. The rank order of Bmax was: acini > total > ducts, indicating that acini express the highest number of binding sites. The specific mAChR antagonists (4-DAMP [M_3], tropicamide [M_4], pirenzepine [M_1]) and the corresponding synthetic peptides impaired IgG-mAChR subtype interactions. The specificity of these reactions was assessed by the corresponding affinity-purified anti peptide antibodies recognizing M_4, M_3 and M_1 mAChR. These data concerning autoantibodies contribute to explain the pathogenesis of SS and also represent a new clinical marker for SS diagnosis.

Sjögren's Syndrome (SS) is a chronic autoimmune disease characterized by histologic and functional alterations of exocrine glands with progressive loss of salivary and lacrimal gland secretion (1-2). The disease may occur as a secondary phenomenon in association with a wide variety of other autoimmune disorders (4), but it also occurs independently as primary SS (pSS).

The criteria for the diagnosis of SS is still controversial (5). In the past, diagnosis depended on the presence of typical clinical features and/or parotid gland swelling together with focal lymphocytic infiltration demonstrated on a biopsy of minor salivary glands and lips. Serological findings have included autoantibodies against SS-A/Ro and SS-B/La (6), anti nuclear antibodies, anti salivary gland antibodies and rheumatoid factor (7-8), although these antibodies have also been linked to other autoimmune diseases.

We have recently proposed a pathophysiological role for circulating anti muscarinic acetylcholine receptor (mAChR) autoantibodies in patients with pSS. These autoantibodies recognized mAChR in both salivary and lacrimal glands (8-9), altering their physiological response (10). Stimulus/secretion dysfunction associated with parasympathetic stimulation have been described in an animal model of SS (11). Moreover, the distribution of IgG antibodies against exocrine glands M_3 and M_1 mAChRs was found to correlate well with SS disease activity (10, 12-13). The finding of M_3 mAChR specific autoantibodies in the majority of the patients (14-15) is an important advance towards understanding, not only the impaired glandular function, but also the parasympathetic dysautonomy in SS patients (7, 9, 12). The detection of antibodies

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against the human mAChR in the sera of pSS patients was demonstrated using a synthetic peptide with aminoacid sequence that represented a part of the second extracellular loop of the human M_3 mAChR and M_4 mAChR.

For this purpose we considered of special relevance to investigate whether circulating mAChR antibodies from pSS patients, not only react and recognize M_3 and M_1 mAChR subtype, but also the M_4 mAChR subtype on the submandibular gland. The data were confirmed by ELISA using two different antigens: submandibular gland membranes (total acini, duct) and synthetic peptides (M_1, M_3 and M_4).

MATERIALS AND METHODS

Subjects and serological test

Women (aged 35-55 years) free of treatment for six months with 5 to 15 years since diagnosis were selected from the metropolitan area of Buenos Aires. The subjects were divided into three groups: group I, 48 primary Sjögren Syndrome (pSS), group II, 35 normal control subjects and group III, 20 rheumatoid arthritis (RA) patients. The diagnosis and serological test of SS, followed four or more criteria of Vitale et al. (15) (labial biopsy, 95% positive; xerostomia, 100% positive; keratoconjuntivitis sicca, 89% positive and two or three serologic test). The serologic test performed were: antinuclear antibodies, 75% positive; anti-Ro, 45,2% positive; anti-La, 40% positive and rheumatoid factor, 30,2% positive. All studies involving human subjects had informed consent and were conducted according to the Declaration of Helsinki.

Total submandibular gland membrane preparations

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires) were housed in our colony in small groups and kept in automatically controlled lighting and uniform temperature conditions. The animals were cared for in accordance with the principles and guidelines of the NIH. Submandibular gland membranes (50 mg/ml) were prepared as previously described (16).

Submandibular acinar and duct gland membrane preparations

Rat male acinar and duct membranes were prepared as described previously (17). Briefly, salivary glands were minced in ice-cold oxygenated Krebs Ringer bicarbonate (KRB) and incubated in 10 ml KRB containing collagenase (2-4 mg/ml) for 40 min in a shaking water bath at 37°C. The glands were dispersed and cells were washed with Ca^{2+}/Mg^{2+}-free KRB as reported (17). The duct cells in the upper phase and the acinar cells in the lower phase were collected. Submandibular gland acini were homogenized for 10 sec twice in 50 mM phosphate buffer, pH 7.4 in an Ultra-Turrax (setting 5). The homogenate was centrifuged for 10 min at 1,000 x g. The pellets were discarded, and the supernatants were centrifuged (10,000 x g) at 4°C for 10 min and then at 40,000 x g for 60 min. The resulting pellets were resuspended in the same buffer (13) and used as a membrane source for the ELISA and binding assays. The purity of the cells was confirmed by microscopic examination before experiments.

Peptides

A 25-mer peptide ERTMLAGQCYIQFLSQPIITFG TAM, a 22-mer peptide QYFVGKRTVPPGECFIQFLSEP and 22-mer peptide QFVVGKRTVPDNQCFIQFLSNP and corresponding to the aminoacid sequence of the second extracellular loop of the human M_1, M_3 and M_4 mAChRs, respectively, were synthesized as previously reported (13).

Purification of Human IgG

The serum immunoglobulin G (IgG) fraction from 48 patients of group I, 35 subjects of group II and 20 patients of group III, was isolated by protein G affinity chromatography as described elsewhere (14). IgG concentrations were determined by radial immunodiffusion assay.

Purification of antipeptide antibodies by affinity chromatography

The IgG fraction of 15 pSS patients and 15 normal individuals were independently subjected to affinity chromatography on the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, Calif.) as describe previously (18).

Enzyme Immunoassay (ELISA)

Fifty microliters of peptide solution (20 mg/ml) or different glandular membrane preparations (3-5 mg/ml protein) in 0.1 M Na_2CO_3 buffer pH=9.6 was used to coat COSTAR microtiter plates at 4°C overnight. After blocking the wells with 2% bovine serum albumin in PBS for 1 h at 37°C, 100 ml of 1/30 dilution of sera of different concentrations or purified IgG from patients of groups I, II and III were allowed to react with peptide for 2 hour at 37°C as reported (18). After 30 min, optical density (O.D.) values were measured at 405 nm with an ELISA reader (Uniskan, USA) (13). As negative controls, non-antigen paired wells and wells with no primary antiserum, were also tested.

Table I. *Inhibition of [^{3}H]-QNB binding to different rat submandibular gland membranes.*

Membrane Preparations	Antagonistic Agents (Ki)			
	4-DAMP	Tropicamide	Pirenzepine	Atropine
Total	1.76×10^{-8} M	6.40×10^{-7} M	5.01×10^{-6} M	4.61×10^{-9} M
Acini	3.71×10^{-8} M	3.62×10^{-6} M	4.22×10^{-6} M	8.02×10^{-9} M
Ducts	5.71×10^{-7} M	3.23×10^{-7} M	6.32×10^{-6} M	7.46×10^{-8} M

The inhibition constants (Ki) for the competing agents were calculated from the equation of Chen and Prussoff: Ki = IC50 / 1 ([^{3}H]-QNB) / Kd, where IC50 is the concentration of competing drug to inhibit 50% of the specific radioligand binding present at a concentration of 0.60 nM on total, acini and ducts submandibular gland membranes. IC50 values were obtained from competition experiments performed in duplicate at several concentrations of each drug.

Radioligand Binding Assay

Submandibular gland acinar membranes were incubated in the final volume of 0.15 ml of buffer Tris-HCl pH 7.4 (buffer A) for 90 min at 37°C with ^{3}H-quinuclydinyl benzilate ([^{3}H]-QNB) (Dupont/New England Nuclear, Boston, MA, USA) with a specific activity of 48 Ci/mmol (1Ci = 37 GBq) with shaking. The reaction was stopped with ice-cold buffer A and filtered through Whatman glass fibre filters (GF/c) under suction. After washing with 12 ml buffer A, filters were placed in vials, dried and counted in

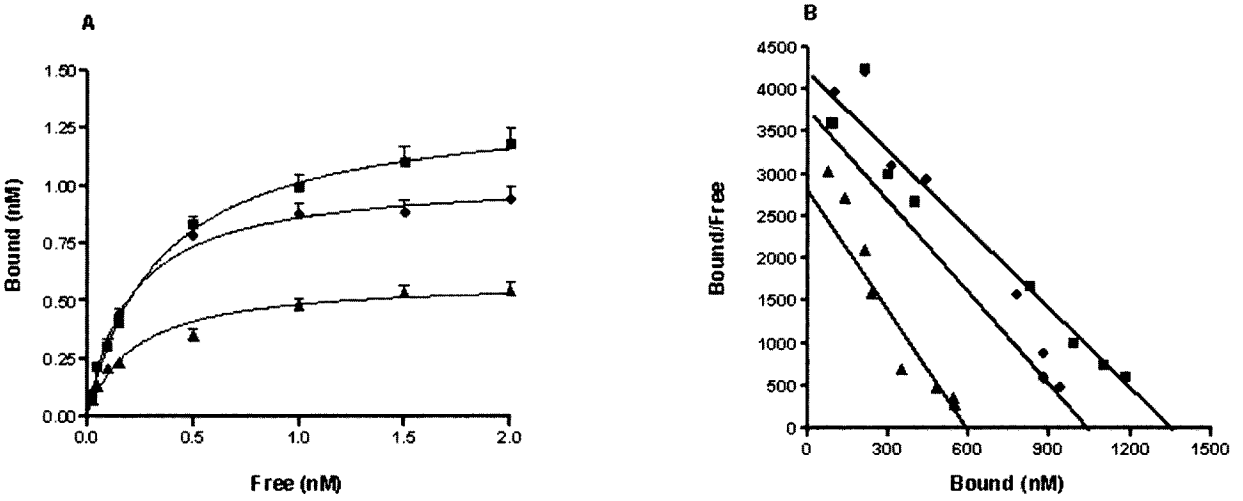

Fig. 1. *Saturation curve and Scatchard analysis of binding assays with [^{3}H]-QNB on rat submandibular gland membranes. Membranes (50 mg protein) of total (●), acini (■) and duct (▲) are shown. Equilibrium parameters calculated from Scatchard Plot were: number of binding sites expressed as fmol/mg protein (Bmax) and equilibrium dissociation constant as nM (Kd) are shown. Results values are mean ± s.e.mean in each group performed by duplicate. This plot is representative of six other experiments.*

A: Saturation Curves.

B: Scatchard Plots.

Table II. *Immunoreactivity of autoantibodies from pSS IgG directed against M_3, M_4 and M_1 mAChR synthetic peptides.*

Affinity-Purified IgG	Optical Density (405 nm)		
	M_3 peptide	**M_4 peptide**	**M_1 peptide**
None	0.15±0.02	0.13±0.01	0.12±0.01
Anti M_3 peptide IgG	1.28±0.08*	0.20±0.02	0.13±0.02
Anti M_4 peptide IgG	0.22±0.02	1.02±0.03*	0.18±0.02
Anti M_1 peptide IgG	0.18±0.01	0.15±0.01	1.12±0.02*
Normal IgG	0.17±0.02	0.15±0.01	0.13±0.02

*Effect of $1x10^{-7}$ M affinity-purified IgG from pSS patients or affinity-purified IgG from normal subjects, against the second extracellular loop of human M_3, M_4 and M_1 mAChR synthetic peptides (M_3, M_4 and M_1 peptides). Values are mean ± s.e.mean of 15 pSS patients and 15 normal subjects in each group evaluated in duplicate. *$p < 0.001$ vs normal IgG.*

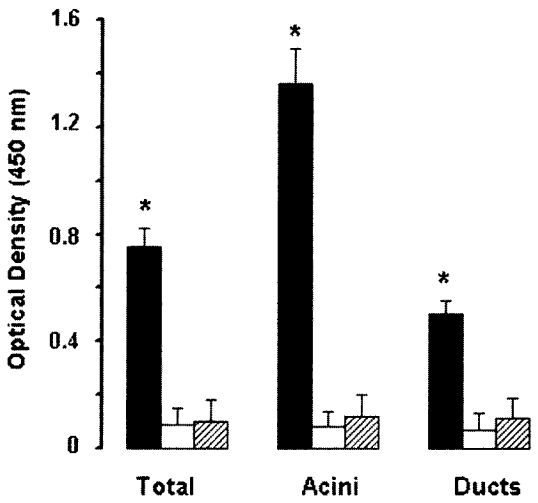

Fig. 2. *Recognition of submandibular gland membranes by IgG of pSS patients. IgGs from 48 pSS patients (black), 35 normal (white) or 20 AR patients (lined) were incubated at $1x10^{-7}$ M in multiwell plates sensitized with 10 µg of total, acini and ducts. Values are mean ± s.e.mean of n patients in each group performed in duplicate. *$p < 0.001$ versus normal IgG.*

8 ml scintillation cocktail (triton-toluene) with about 40% efficiency. Non specific binding (measured in the presence of 10 mM atropine) did not exceed 15%. For competition assays, 100 ml of the membrane solution (50 mg protein) was used for the binding assays with 0.6 nM [^{3}H]-QNB. For saturation assay, membranes were incubated with different concentrations of [^{3}H]-QNB (0.1-1.5 nM). The equilibrium dissociation constant (Kd: nM) and the number of binding sites (Bmax: fmol/mg protein) were taken from plots according to the methods of Scatchard (19).

Drugs

4-DAMP, pirenzepine and tropicamide were purchased from Sigma Chemical Company, Saint Louis, Mo, USA. Stock solutions were freshly prepared in the corresponding buffers.

Statistical Analysis

Student's t-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) was employed when a pairwise multiple comparison procedure was necessary. Differences between means were considered significant if $P < 0.05$.

RESULTS

The radioligand competition binding assay was carried out on total, acini and ducts submandibular gland membranes. Binding of the specific radioligand [^{3}H]-QNB to submandibular gland membranes was a saturable process to a single class of binding sites. Fig. 1 shows linearizations of saturation assays for [^{3}H]-QNB performed in total,

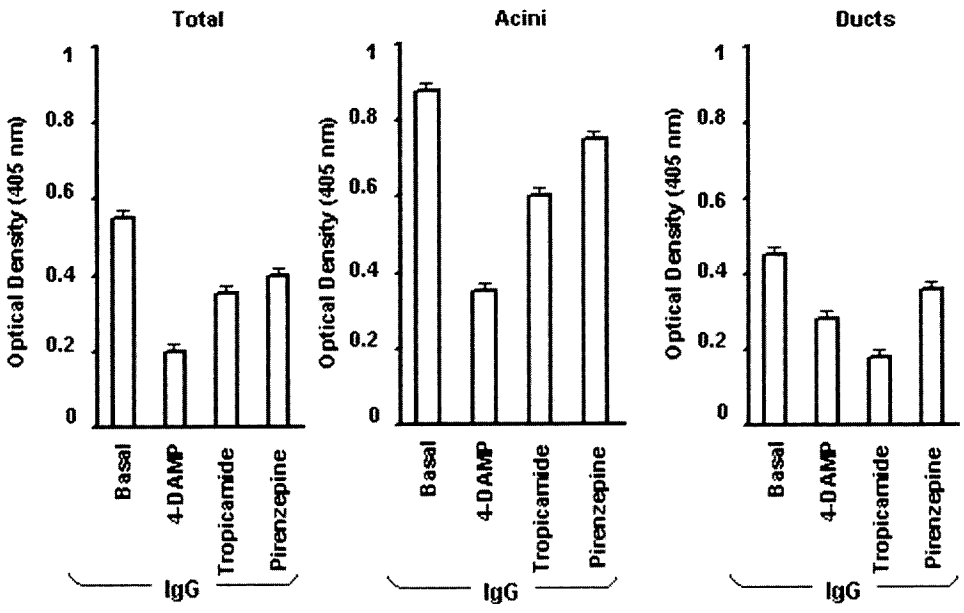

Fig. 3. *Immunoreactivity of IgG antibodies from pSS patients (1x10-7 M) directed against total, acini and ducts treated with 1x10-7 M 4-DAMP, tropicamide and pirenzepine tested by ELISA. Basal refers IgG alone. Values are mean ± s.e.mean of 48 pSS patients in each group evaluated in duplicate.*

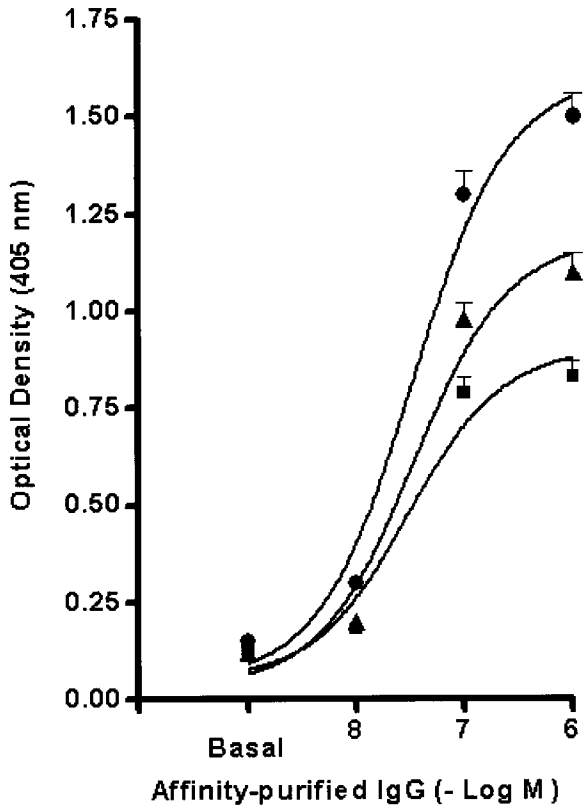

Fig. 4. *Immunoreactivity of affinity-purified anti M_3 (●), anti M_4 (■) and anti M_1 (▲) mAChR antibodies against total submandibular gland membrane preparations tested by ELISA. Effect of increasing concentrations of affinity-purified anti M_3, anti M_4 and anti M_1 peptide IgGs on gland membrane preparations (50 µg/ml) used as coating antigens. Values are mean ± S.E.mean of 15 pSS patients in each group evaluated in duplicate.*

acini and duct membranes. The equilibrium dissociation constant (Kd) was not statistically different between the three glandular membrane preparations: total: 0.20 ± 0.017 nM; acini: 0.33 ± 0.023 nM and ducts: 0.22 ± 0.040 nM. The maximal number of binding sites (Bmax, fmol/mg protein) for the three different preparations was statistically different: total, 1038 ± 24, acini, 1359 ± 28 and ducts, 593 ± 30. The rank order of B_{max} was: acini > total > ducts indicating that acini express the highest number of binding sites.

Rank of Ki values calculated from [^{3}H]-QNB competitive binding assay using specific mAChR subtype antagonists showed: acini, $M_3 > M_4$ and ductus, $M_3 = M_4$. Similar Ki values to M_1 in all preparations were observed (Table I).

Detection with an ELISA assay using total, acini and ducts as coating antigens, we demonstrated the presence of anti submandibular gland autoantibodies in the sera of pSS patients. Figure 2 shows increase in O.D. values with the IgG from pSS patients with O.D. values stable at > 3 SD from normal subjects, acini values being the highest. No differences were observed between O.D. values from RA patients and normal subjects (Fig. 2).

To know the ability of the autoantibodies from pSS patients to recognize different mAChR subtypes, ELISA was performed using total, acini and ducts treated with specific cholinergic subtype

antagonists (4-DAMP for M_3, tropicamide for M_4, pirenzepine for M_1) as coating antigens. Figure 3 shows that 4-DAMP was more effective to block O.D. values in total and acini membranes than in ducts. On the other hand, tropicamide was more effective on ducts than on total and acini membrane preparations. Pirenzepine has no statistically differences in its ability to block O.D. values among preparations. These results point to the capacity of IgG from pSS patients to recognized M_3, M_4 and M_1 mAChRs on the submandibular gland, revealing significant differences between the three submandibular gland preparations (Fig. 3).

Moreover, the IgG from pSS patients recognized human M_3, M_4 and M_1 mAChR synthetic peptides, confirming the presence of anti M_3, anti M_4 and anti M_1 autoantibodies in the sera of pSS patients. Figure 4 shows the concentration-dependent increase in O.D. values with the corresponding affinity-purified IgG, using total submandibular gland membranes as coating antigens. The maximal O.D. values obtained with the affinity-purified antibodies were $M_3 > M_4 > M_1$, always 3 > SD of those from normal individuals treated as pSS patients.

The specificity of the anti M_3, anti M_4 and anti M_1 peptide IgGs was revealed by the ability of the M_3, M_4 or M_1 synthetic peptide (10 fold concentrated) to inhibit the reaction when affinity-purified anti M_3, anti M_4 or anti M_1 peptide IgG were incubated with the corresponding peptide for 30 min at 37°C and then, added in the microtiter plates. The O.D. values for each IgGs from pSS and normal individuals studied are shown in Table II.

DISCUSSION

Here we present evidence that IgG from patients with pSS recognizes and interacts with M_3, M_4 and M_1 mAChR subtypes expressed on rat submandibular glands. The observed ability of pSS circulating autoantibodies to specifically recognize peptides homologous to an extracellular domain of the human M_3 and M_1 mAChR subtypes, confirmed previous results (7-13). But, we considered of special relevance that the autoantibodies from pSS could act also on M_4 mAChR subtype of submandibular gland and whether specific cholinoceptor antagonists are able to interfere with the immunoreactivity of pSS IgG with mAChR subtypes.

Our findings demonstrated that M_4 mAChR subtype is expressed in submandibular gland together with M_3 (16) and M_1 (10) subtypes. Differences in mAChR subtypes expression were observed on the three preparations studied. As expected, in total and acini glandular membranes, M_3 is the most expressed, M_4 was lower than M_3 and M_1 was less expressed on all preparations.

On the contrary, M_4 mAChR subtype was preferentially expressed in duct membranes. The pSS IgG revealed the capacity to interact and recognize the M_4 mAChR in all preparations tested. Moreover, the differential SS IgG-interaction with M_3, M_4 and M_1 mAChR subtypes is related with the differential expression of each subtype receptor on total, acini and ducts. The specificity of these interactions were assessed by the fact that the corresponding affinity-purified anti peptide antibody behaved similarly to total SS IgG.

Altered humoral immunity from pSS patients, actually contribute to the pathogenesis of parasympathetic dysautonomy present in pSS (20) and represents a new clinical marker for SS diagnosis.

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